Synthesis of lignin star model compounds and their dehydrogenation polymer growth study.

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Tiivistelmä/Referat – Abstract

Lignin synthesis has been debated for quite some time, it is uncertain whether the polymerization is simply chemically and kinetically controlled or if other entities, such as enzymes and proteins, are involved. If lignin synthesis is only chemically controlled, then some of the polymer growing branches will eventually react together. When two growing chains, already linked at a branching point, react together, a closed loop is created. The purpose of this study is to look for these closed loops. As lignin structure is complicated and the amount of branching points not too elevated, a model is needed to simplify the problem.

The most employed model for lignin is dehydrogenation polymer, obtained from radical oxidation of coniferyl alcohol. In order to have a chance of detecting the closed loops in the model polymer it is necessary to increase the number of branching points. The idea to achieve this goal came from star polymers, where a multibranched polymerization center is used to start the growth of polymeric chains in multiple directions. The intoduction of a multibranched polymerization center in the dehydrogenation polymer should provide the necessary amount of branching points and increase the chances of closed loops detection.

Three new, three-branched model compounds were synthesized, to act as polymerization centers. The synthesis of these compound had never been tried before. Two of the model compounds were synthesized and characterized, but the reaction yield for one of them was too low to proceed with the study. the synthesis of the third model compound was abandoned. Coniferyl alcohol polymerization tests have been run with one of the model compound and with two model compounds precursors. This research is at a too early stage for the detection of closed loops, but some encouraging results have been obtained in that direction.

Avainsanat – Nyckelord – Keywords

Lignin, DHP, model compounds, coniferyl alcohol, NMR, SEC,

Säilytyspaikka – Förvaringställe – Wheredeposited

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List of Abbreviations

4CL = 4-Coumarate CoA ligase

BBAVone = 1-(4-(benzyloxy)-3-methoxyphenyl)-2-bromoethan-1-one

C4H = Cinnamate-4-Hydroxylase

CCOMT = Caffeoyl CoA O-Methyltransferase

CCR = Cinnamoyl CoA reductases

CM = Central Molecule (molecule present in polymerization reaction mixture)

CoA = Coenzyme A

COMT = Caffeic acid O-Methyltransferase

DEPT = Distortionless Enhancement by Polarisation Transfer

DFRC = Derivatization Followed by Reductive Cleavage

DHP = dehydrogenation polymer

DIPA = Diisopropylamine

DMF = dimethylformamide

DMSO = Dimethyl sulfoxide

DP = Dirigent Protein

EtOAc = Ethyl Acetate

F&F = Flavors and Fragrances

F5H = ferulate-5-hydroxylase

FAME(s) = Fatty Acid Methyl Ester(s)

FTS = Fischer-Tropsch Synthesis

G = guayacyl phenylpropanoid unit

GC = Gas Chromatography

GTL = Gas to Liquid

H = p-hydroxyphenyl phenylpropanoid unit

HCT = hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase

HMBC = Heteronuclear Multiple Bond Correlation

HPLC = High Performace Liquid Chromatography

HQT = hydroxycinnamoyl CoA:quinate hydroxycinnamoyl transferase

HRP = Horseradish Peroxidase

HSQC = Heteronuclear Single Quantum Coherence spectroscopy

LC = Liquid Chromatography

MC 1, 2, 3 = Model compound 1, 2, 3 .

MeRe = MethylResorcinol

$MeRe(BAV)_2$ = 2,2'((5-methyl-1,3-phenylene)bis(oxy))bis(1-(4-(benzyloxy)-3-methoxyphenyl)ethan-1-one)

MG = coniferyl alcohol, monomer

MH = p- coumaryl alcohol, monomer

M_n = number-average molecular weight

MS = Mass Spectrometry

MS = synapyl alcohol, monomer

Mw = Molecular Weight

M_w = weight-average molecular weight

MWL = Milled Wood Lignin

NADPH = Nicotinamide Adenine Dinucleotide Phosphate

n-BuLi = n-Butyl Lithium

NMR = Nuclear Magnetic Resonance

NOESY = Nuclear Overhauser Effect spectroscopy

PAL = Phenylalanine Ammonia Lyases

pC3H = p-coumarate-3-hydroxylase

PDI = Polydispersity

Ph(OBAVone)3 = 2,2',2''-(benzene-1,3,5-triyltris(oxy))tris(1-(4-(benzyloxy)-3-methoxyphenyl)ethan-1-one)

$Ph(OH)_3$ = Phloroglucinol

Phe = Phenylalanine

RI = Refractive Index

RSCL = Released Suspension cell Culture Lignin

RT = Room Temperature

S = syringyl phenylpropanoid unit

S1 = inner layer, secondary cell wall

S2 = middle layer, secondary cell wall

S3 = outer layer, secondary cell wall

SEC = Size Exclusion Chromatography

TAL = Tyrosine Ammonia Lyases

TOCSY = TOtal Correlated SpectroscopY

Tyr = Tyrosine

WGS = Water-Gas Shif

1 Introduction

It does not matter who you listen to or which article you read, everybody agrees that we are running out of fossil fuels. It might be sooner or later, according to different evaluations, but oil and coal will inevitably end and probably in a not so distant future [1]. The current industrial development is unsustainable, both from an energetic and an environmental point of view. We are using too many resources, we are exploiting the territory too much, and we are so focused on the present that we do not care about the future. During the past century energy consumption has increased 17 folds with related emission of greenhouse gasses CO_2, SO_2 and NO_x [2].With the world population hitting 10 billion in 2050 [3], this trend is clearly self-destructive. More and more people, in and out of the scientific community are becoming aware of that, and the requests for a greener and more sustainable way are becoming more relevant.

Politics will be left aside; it is beyond our purpose to say which country is doing good or bad and why. It is better to focus on what science, and chemistry in particular, can do to help. Even if chemistry is usually seen as dangerous, poisonous and polluting, this is only one of the sides of the medal. Of course chemical industry has been responsible for ecological disasters, even more so if oil spills and nuclear accidents are included in the count. The media impact of these catastrophes is huge, which explains chemistry bad reputation. The other side of the medal is unknown to most people, for example the study to develop greener and safer procedures, the search for eco-friendly materials and reactants, and the creation of new and more sophisticated devices to reduce pollution. These are all things that chemistry is involved into, and hopefully in the future the image of chemistry will change to reflect that as well.

One of the possible solutions to the environmental problems that afflict the earth, are renewable resources. Everything necessary for our society, such as energy and chemicals, should be produced in a sustainable way, without using more that what the earth can produce. Otherwise our “ecological capital” will soon be exhausted and there will be nothing left. There are several renewable resources that are currently being discussed and researched such as solar, geothermal, wind and hydroelectric power. The aim of this paper is not to review them all, but to focus on the conversion of biomass. Biomass conversion and employment is the wider topic in which the small and specific research of our group belongs.

1.1 Biomass

Biomass is the biological material derived from living, or recently living organisms [4]. This includes any kind of plant grown for any purpose, like converting them to fuels or chemicals, and all sorts of bio wastes such as municipal and agricultural wastes or forest residues. Biomass, as a source of energy, is much more evenly distributed than fossil fuels; therefore country investing in biomass conversion will not only help the environment but will also decrease their energy dependency from foreign sources. As far as renewable resources go, biomass is unique and different from all the others. The reason is that biomass is the only renewable resource that produces carbon, which means it is the only currently known way to sustainably produce chemicals and fossil-like (carbon-based) fuels. It does not mean that biomass is the best of all renewable resources, because these advantages come with severe drawbacks. In fact, biomass has a much lower energy production potential if compared to solar, and it is usually competing with food production, either by processing edible resources or by occupying agricultural terrain. Advantages and disadvantages of biomass will be discussed later, after an overview of the possible applications. The first topic treated will be conversion of biomass to fuels, followed by conversion to chemicals.

1.1.1 BIOMASS TO FUELS

There are several ways to convert biomasses to biofuels, depending on the kind of biomass employed, the different catalysts, the process adopted and the kind of fuel. The most common, promising and state of the art technologies for conversion will be shortly reviewed.

Thermal conversion.

Beyond old fashioned wood combustion, there are two main thermal treatments currently performed on biomass: gasification and pyrolysis. Gasification is a fast procedure conducted at high temperature, optimized to increase the yield of the gaseous fraction. The process produces low amount of solid residues, and the composition of the gaseous part can be tuned by modifying the reaction atmosphere. Air or pure oxygen will increase the amount of CO_2, while steam increases the H_2 yield. Gasification is currently the most promising way to thermally convert biomass [5,6]. Pyrolysis is conducted under more

moderate conditions: lower temperature, longer residence time, higher pressure, low concentration of oxygen. The main product is pyrolysis oil, a liquid mixture of more than 400 small molecules, most of them hydrocarbons [7,8], with both a solid and a gaseous fraction as byproducts, which are usually burned in place in order to produce the heat necessary for the reaction [9,10].

For the conversion to fuels, both pyrolysis and gasification products, undergo steam reforming to produce bio syngas, a mixture of CO and H_2 [11]. During steam reforming the hydrocarbons react with steam, with the help of a nickel-based catalyst [12], according to:

$$C_nH_m + nH_2O \rightleftharpoons nCO + (n+m/2)H_2 \qquad \text{Formula 1.1}$$

This reaction commonly employs methane as feed. Substitution of this high purity reactant with biomass feeds makes the process harder, requires optimization and additional purification steps. This translates in higher costs and lower yield compared to fossil-based processes [13]. It is to be said that currently steam reforming of CH_4 is the most used and economically favorable way to produce pure H_2. It involves two water-gas shifts (WGS) steps, CO purification and CO_2 removal. In principle the methane could be substituted by biomass, but the yield would be extremely reduced. A lot of research is necessary to match the yield of bio-based processes with the yield of petrol chemistry. Besides, hopefully in the future H_2 will be produced by water electrolysis and therefore the whole biomass to hydrogen technology will not be needed.

Once a pure feed of syngas is obtained, it can be easily converted to fuels through the available and economically viable technology of Fischer-Tropsch Synthesis (FTS). FTS has been around since the 1930's, when it was introduced in Germany [14], a lot of progress has been made ever since and there is a wide and flourishing literature on the subject [15-18]. FTS converts syngas to linear olefins, a high quality fuel. The feed needs to be pure in order not to poison the catalyst, resulting in a cleaner product, without S, N or aromatic impurities. This makes fuels produced by FTS more eco-friendly than the petroleum-based ones. The catalysts employed are iron-based with Al_2O_3/SiO_2 doping. By variation of the doping and reaction conditions the distribution of the products can be controlled, it is possible to statistically obtain the desired length of hydrocarbon chains to produce gasoline, kerosene and diesel. It is evaluated that as the price of oil rises, FTS-based gas to liquid (GTL) technology will become more and more important [19].

To summarize, it is possible to convert biomass to high purity carbon-based fuels. The process requires first conversion of biomass to hydrocarbons by gasification or pyrolysis. Then steam reforming of the obtained hydrocarbons to produce syngas and eventually synthesis of gasoline and diesel by FTS.

Bioalcohols: methanol, ethanol and butanol.

Bioalcohols are a category of bioproducts that can be, and currently are, employed as clean liquid fuels. Ethanol (**2**) is the most important of the three and will be discussed longer. It is very similar under many aspects to butanol (**3**), therefore the latter will be treated very shortly. On the other hand methanol (**1)** production is very different from the other two and will require a separate treatment. The mentioned alcohols are reported in Figure 1.1.

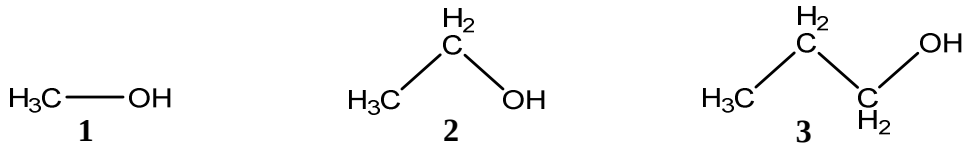
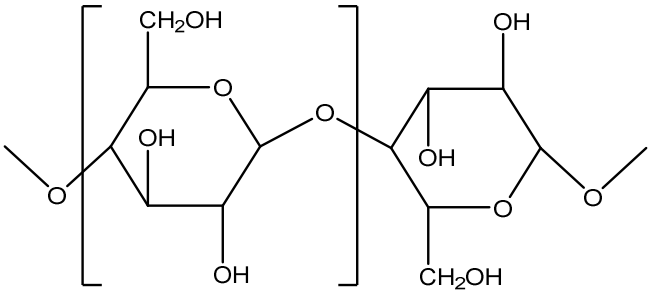


Figure 1.1Methanol, ethanol and butanol, the alcohols most employed as biofuels.

Ethanol is produced trough biological fermentation of sugars, usually using yeast, and then distilled to the purity required by engines. It can be used as an additive to standard fuels without engine modification or can be burned pure in specific devices. When used as additive to gasoline it increases the octane number and the oxygen content, facilitating combustion [20]. The cost for ethanol production is not currently competitive with oil-based fuels, but it is made so by governmental support in several countries [21]. Corn, wheat and sugar beet are common feedstock for bioethanol production, with good yields and favorable conversion.

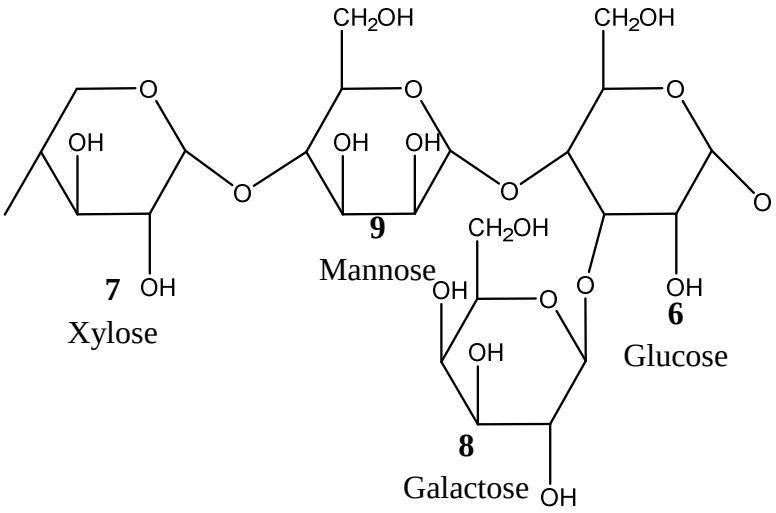
As favorable as it may be, ethanol from corn is in competition with food production, and causes the rise of food prices and necessitates terrains that cannot be dedicated to other cultures. Therefore it is necessary to find an alternative to fermenting ethanol from edible feedstock. Lignocellulosic materials, such as wood and agricultural wastes, are a viable alternative, but there are several connected problems that need to be solved. Wood and grasses are composed mostly of cellulose (**4**), hemicellulose (**5**) and lignin. Cellulose is a $\beta(1\rightarrow4)$ linked D-glucose (**6)** homopolymer, a linear polysaccharide hundreds to thousands unit long, non soluble in water. It is the most abundant biopolymer on earth. Cellulose

content in wood is about 45% and it is mostly crystalline. Hemicellulose is a heteropolymer of xylose (**7**), galactose (**8**), mannose (**9**) and other sugars. It is a branched amorphous polysaccharide [22]. Figure 1.2 shows the structures of cellulose and hemicellulose.



6 Glucose **6** Glucose

4 Cellulose



5 Hemicellulose

Figure 1.2 Cellulose (above) is a linear β(1→4) linked D-glucose homopolymer, while Hemicellulose (below) is branched and composed by more than one monomer. The structure of some of the monomers is shown.

Both cellulose and hemicellulose are composed by monosaccharides and therefore can be used for ethanol conversion. Lignin is an aromatic compound encrusted around cellulose, it is very hard to eliminate, in order to obtain pure cellulose and hemicellulose. Lignin composition, applications and related problems will be extensively discussed later (p. 23). For the current purpose is enough to know that cellulose purification is not an easy task, and requires expensive treatments. Once lignin is eliminated, it is necessary to hydrolyze (depolymerize) cellulose and hemicellulose to obtain monosaccharides. Hydrolysis can be obtained by chemical or enzymatic process. Chemical hydrolysis is obtained after reaction

with acid. The acid concentration influences the outcome, but both diluted and concentrated acid hydrolysis have some advantages. Enzymatic hydrolysis is achieved with specific enzymes that cleave the bonds between polysaccharides [13].Once the hydrolysis is complete it is possible to convert monosaccharides to ethanol trough simple fermentation. Specific description of each method is beyond the purpose of this review. Butanol is processed in the same way as ethanol, with the exception that it is necessary to find a different organism to convert the sugars. Butanol has several advantages over ethanol, in particular: Butanol can be blended in higher amount with gasoline, it has a higher octane number, can be transported in existing pipelines and provides better safety [23].Methanol has similar characteristics as a fuel as the other alcohols, but is different when its production is concerned. Sustainable methanol production is currently not economically possible. Methanol is normally synthesized from syngas, but could also be obtained in the future from coal, after cleaning and reaction with steam and O_2. To convert biomass to methanol it would be necessary to first convert biomass to syngas with the process described above (p. 10) and then use the syngas to produce methanol. This long process makes agricultural methanol way more expensive than bioethanol and methanol from natural gas [24].

Biodiesel

Triglycerides (**10**) extracted from oleaginous plants, such as soybean, rapeseed and sunflower, were tested as diesel fuels by Rudolph Diesel more than 100 years ago. They turned out to be far too viscous to be employed, causing clogging, and with a too low cetane number. Triglycerides are fatty acid esters of glycerol (**11**), with a carbon chain of 12 to 18 units, and they can be easily transesterificated with methanol (**1**) or ethanol (**2**). The fatty acid methyl esters (**12**) (FAMEs) obtained, have the right viscosity and properties to be employed as biodiesel. No engine modification would be required, and since they are obtained from biomass their overall CO_2 production would be near 0, making them a good green alternative to diesel. As a bonus, transensterification from triglycerides to FAMEs gives pure glycerol as a byproduct. Currently glycerol is employed mostly in pharmaceutical and cosmetics industries but the huge amount of glycerol produced by a FAMEs fuel industry would easily saturate the market.

The traditional way to transesterification is via liquid basic catalysis [25], but as solid catalysts are much better for industries, several studies have been made in that direction [26]. Strong organic bases or enzymes, immobilized in zeolites or polymers, as well as

multimetallic oxides catalysts have been studied with various degree of success [27-30]. Figure 1.3 shows the general reaction of triglyceride transesterification to FAME.

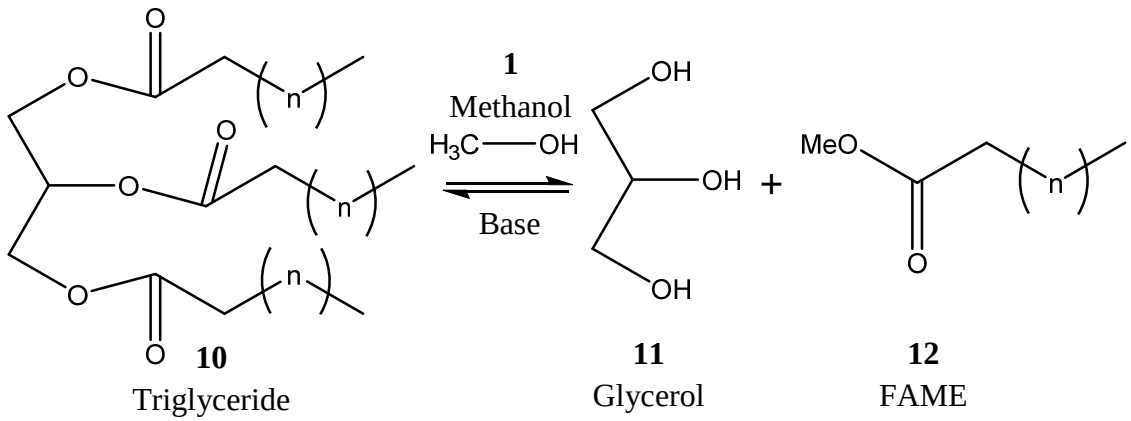


Figure 1.3 Shows the transesterification reaction of a triglyceride and methanol, to produce FAMEs.

Beyond the technological problems related to the conversion from glycerol to methyl esters there are several obstacles preventing FAMEs to become an economically viable fuel. First of all, the price of the feedstock makes the cost of biodiesel to be the double of petrol derived diesel. The second problem is more ethical than economical: every plant that could be used to extract fatty acids is also edible. It would be enough to use waste cooking oil, beef tallow and yellow grease, but again all the problems related to feed impurities will have to be solved, and new processes and catalysts would have to be researched and optimized.

Conclusions

Biomass as a source of fuel, and thus energy, shows great advantages but also big limitations. First of all, biofuels are in principle CO_2 neutral. All the CO_2 produced by combustion, has been absorbed from the atmosphere by the plants, which makes them so relevant in green chemistry. Biofuels are also sustainable, as they come from a renewable resource. Those two last points are more or less common to all other renewable energy sources, but what makes biomass unique is the chance to produce liquid carbon-based fuels similar to petrol-derived ones. This means that the change of feedstock, from fossil to renewable, would not require a radical change in the existing industries and technologies. It is much easier to introduce something on the market if you do not have to modify and reinvent every single process or instrument. Moreover, the possibility to produce fossil-like

fuels means that it is possible to efficiently store energy derived from a renewable resource, which is not possible yet for all the others (at least until a viable way to electrolyze water to hydrogen and store it is found). Finally, it is necessary to discuss the effective gain in energy that biofuel could provide, i.e. the energy balance between the energy necessary to produce biofuels and the energy provided by the same biofuels. The subject is thoroughly discussed by Hill et al. and the outcome is that the balance is positive, even if not outstanding [31].

Biofuels sound great, but after the advantages it is necessary to give a good look to the drawbacks. Even with state of the art technologies and catalyst, there isn't any process that can be cost-competitive with fossil fuels. Every biofuel on the market can be afforded only thanks to government support. It is most likely that, as the oil price rises and new technologies lower the biofuel price, this situation will be eventually inverted.

Even with the most optimistic evaluations, biomass-based fuels will never be enough to cover the world's energy demand. There are different assessments and different opinions, with the same core message: biomass potential for energy production is not enough and other renewable resources will be necessary to fill that gap. In 2005 a report from USDA estimated the amount of all agricultural and forest wastes at about 1.350 billion tons, calculated to satisfy 30% of fuel transportation demand in the US [23]. Again in 2005 it was calculated that devoting all US corn and soybean production would only cover 12% and 6% of gasoline and diesel consumption, respectively [31].This introduces the ethical problem concerning biomass conversion. The highest yield for biofuels production is obtained by conversion of edible feedstock. Leaving aside every economic consideration (food prices rising and the likes) I do not think it would be proper to throw food in the tank when people are starving. On the other hand, when wastes are used in place of edible feedstock, the situation is inverted. It is a good thing to be able to recycle wastes, which would maybe otherwise pollute, into something useful; but using wastes as feedstock has its downsides, mostly connected to the impurities present in the reactant. This lowers the yield and requires more research to optimize the process. Another downside of wastes is that they are usually a huge amount of matter with really low value and low production potential. Therefore, transport of wastes is a relevant expense in the production process. In order to decrease the transportation cost, wastes need to be treated in situ, to increase the value/mass ratio. Pyrolysis or drying of agricultural wastes, prior to transportation to the conversion plant, would increase the value of wastes.

An alternative to a waste-based industry is to grow plants for the specific purpose of converting them to fuels. This should be done with non-edible plants in non-agricultural terrain, such as seaweeds, or trees in non productive fields. Hopefully in the future it will be possible to actively produce energy from wastes and non food competitive cultures.

1.1.2 BIOMASS TO CHEMICALS

Conversion of biomass to chemicals is a more complicated topic than conversion to fuels. Even if the amount of raw material converted to chemicals is only a small fraction of what is employed for fuel production, the wide range of target molecules, the variety of processes and strategies and the difference in reactants makes the topic broader and harder to summarize. At present three main categories of biomass-based materials are used for chemicals production: carbohydrates, vegetable oils and, in much lower quantities, terpenes. Big efforts are being made to include lignocellulosic materials in this list, but so far only few processes are available [32].

Also strategies for conversion can be divided in three wide areas, the first being via degraded molecules. Conversion via degraded molecules means that biomass is either gasified to syngas or pyrolyzed to bio-oil. Those starting materials are then converted using the standard oil industry processes. Hydrocarbons are made out of syngas and pyrolysis oil is separated, fractionated and reacted to obtain standard petrol chemistry building blocks. This is a waste of biomass potential, as complex and highly functionalized molecules are reduced to small building blocks in order to rebuild complex molecules after several steps. It is currently the most common way to convert biomass because it does not require new synthetic routes or the study of new processes. Some really interesting studies have been made in this field [33]. Such studies are necessary to promote biomass culture and research. It is not realistic to think that, from one day to another, it will be possible to abandon all the oil-based plants and rebuild new biomass-based ones. Therefore is necessary to apply the old synthesis method to the new resource, at least for a while. It goes by itself that this strategy will hardly be cost-effective. Energy is employed to convert biomass to small and cheap molecules easily obtainable from petrol distillation.

A second approach to biomass conversion is via platform molecules. This concept is much better suited for the complete employment of biomass potential. The general idea is to treat biomass in a biorefinery, i.e. a facility where biomass is reacted to obtain fuels, power, heat and valuable chemicals just as it happens in a modern oil refinery. This strategy requires

the study of a completely new kind of chemistry, which starts from new building blocks and requires new processes and new catalysts. Of course there is a huge amount of work, but it is the way to fully exploit biomass. Big efforts are made to promote the biorefinery concept, and several new platform molecules have been proposed [34].

The third and last kind of approach is via one-pot reactions. It is even harder to achieve than the biorefinery strategy. Commercial products are usually a mixture of different chemicals. It is possible to directly convert the different component of biomass in a single-pot reaction including several catalytic steps, and obtaining a commercial product. This way most separation and purification steps would be skipped, reducing production cost and pollution. Of course the design and optimization of a one-pot process is extremely hard, but still some research in that direction has been made [35-38].

Before a short overview of relevant applications, a few important points about chemicals from biomass should be underlined. According to current knowledge, to achieve a fully sustainable society there is no way around conversion of biomass to chemicals. Even if it is possible to obtain energy from other renewable resources, such as solar, using biomass as feedstock is the only way to sustainably produce carbon and therefore chemicals. Another advantage is that materials and products derived from biomass are usually biocompatible, for example biodegradable polymers. Therefore conversion of biomass to chemicals does not only help achieving sustainability but also reduces pollution.

Conversion of triglycerides

As explained before, triglycerides (**10**) are fatty acid esters of glycerol (**11**). Their physical properties make them suitable as a substitute for lubricants. About 50% of lubricants leak and are spread in the environment, therefore a biodegradable alternative to petrol-derived lubricants would help reduce pollution. FAMEs (**12)** are better suited than triglycerides as lubricants (their synthesis is described above, p.14) but still their oxidation resistance and rheological properties need to be improved. This can be achieved by epoxidation of the FAME followed by alcoholysis [39]. Traditionally epoxidation is performed with strong acid liquid catalysis. Recently researches managed to make the process greener and more economically favorable for industries by introducing a solid catalyst. Titanium supported catalyst gave good yields and selectivity [40]. Alcoholysis is performed using methanol (**1**) and acid resins. The reacted fatty acids proved to be suitable as lubricants and biodegradables [41].

Further applications for triglycerides include conversion to surfactants and polymers [42,43]. A related topic is the conversion of glycerol, produced after transesterification of triglycerides, to more useful chemicals [44,45].

Conversion of carbohydrates

Carbohydrates are the largest amount of biomass-based material converted to chemicals, mostly coming from two sources, sucrose and starch. Carbohydrates conversion is a wide and complicated topic; it is really hard to find a suitable example to summarize every possible application. Carbohydrates are mostly employed in food and pharmaceutical industries, chemical modifications are required to fit carbohydrates to the needs of industry. Instead of focusing on a single example, the most common chemical reactions employed and their particularities will be shortly described.

Hydrolysis of carbohydrates to obtain monosaccharides is probably the most common reaction in this field [46]. Carbohydrates are easily hydrolyzed under mild condition, and they do not present the problems described above for cellulose (**4**) (p.12). Once monosaccharides, mostly glucose (**6**), are obtained, a common reaction is hydrogenation to obtain mannitol (**13)** and sorbitol (**14)**, with glucuronic acid (**15**) as a byproduct. As they are employed in fine chemical industries, high yield and selectivity are required. Most production is still based on Raney-Nickel catalysis, but ruthenium or platinum/ruthenium catalysts seem to give good results [47]. Another interesting reaction is dehydroxylation (C-OH bond breaking). The purpose is to produce deoxyhexitols (**16)** from sorbitol, which can be used for polyester and polyurethane production instead of petrol derived molecules. Copper-zinc catalysts are used to obtain deoxyhexitols while Palladium catalysts produce isosorbide (**17**) [48]. Finally, carbohydrate oxidation is widely employed to obtain added value chemicals, such as vitamin C (**18**). Most reactions are carried out with homogeneous, enzymatic or microbial catalysis [49]. Figure 1.4 shows some examples for the reactions described above. Despite all the possible application seen so far, carbohydrates present an even more important feature. In fact most of the molecules proposed as biorefinery building blocks are derived from saccharides fermentation. Levulinic acid (**19**), succinic acid (**20**), 2-hydroxypropionic acid (lactic acid) (**21**), and 3-hydroxypropionic acid (**22**) are all products of carbohydrate fermentation and they are currently the focus of a lot of studies. Hopefully, in the future, those will be the platform molecules that will sustain chemical industry [34,50-52]. Figure 1.5 shows the proposed future biorefinery building blocks.

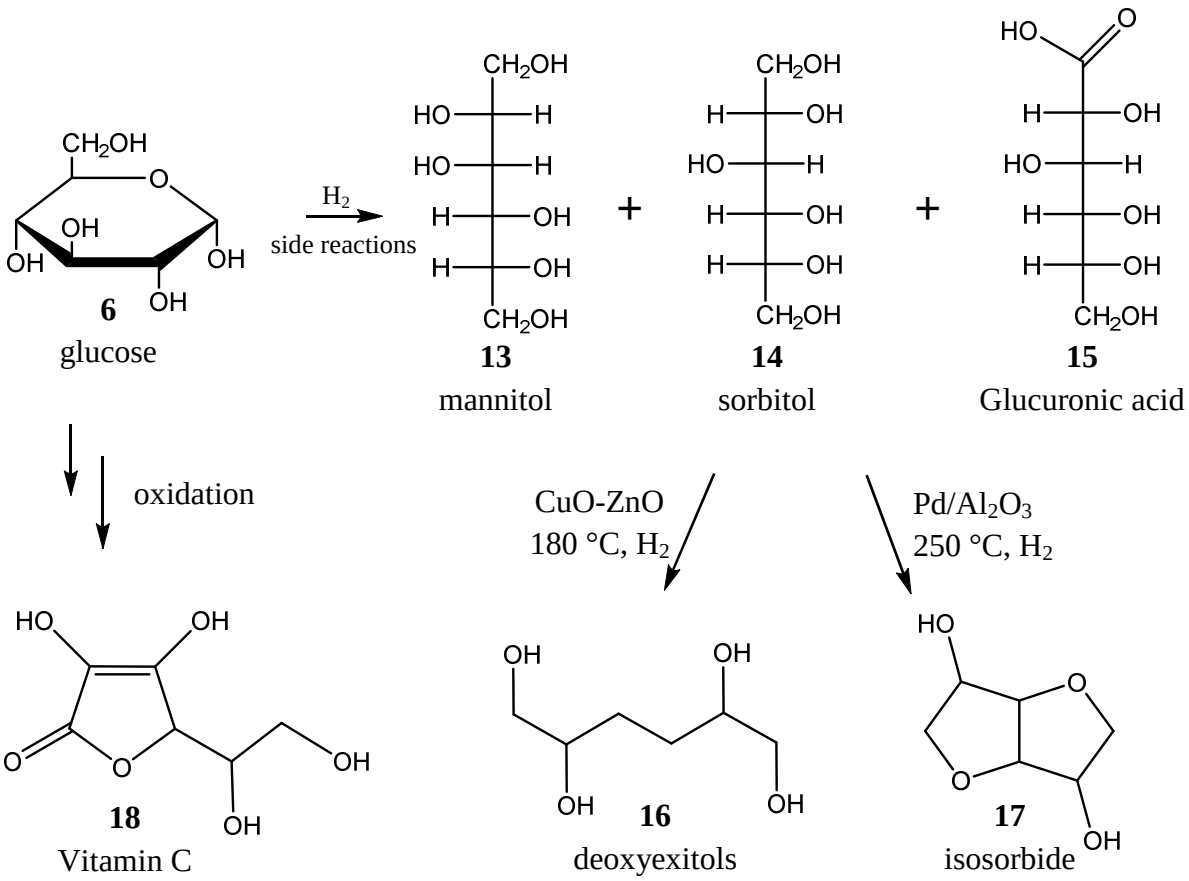


Figure 1.4 shows just some of the most common industrial reaction conducted on glucose.

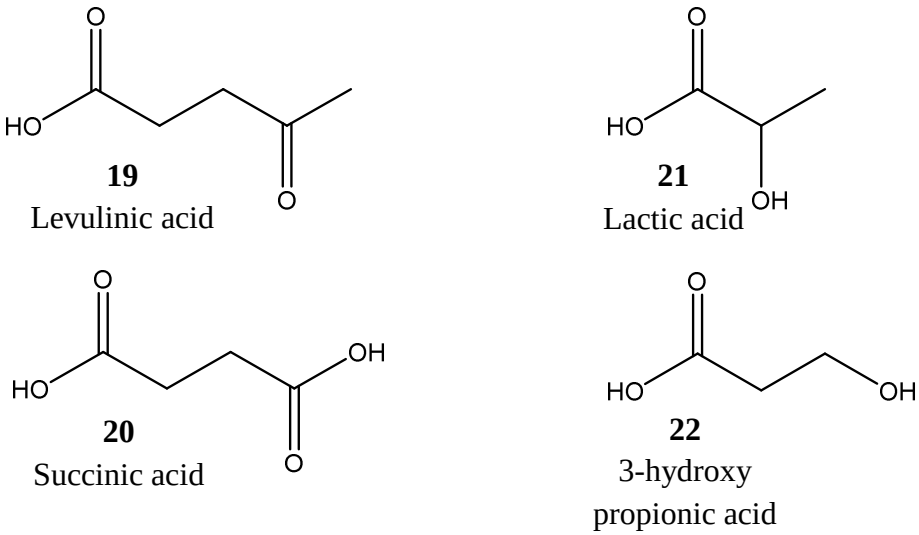


Figure 1.5 shows some of the most promising building blocks and platform molecules for a future biorefinery. The proposed are easily produced by sugars fermentation.

Terpenes conversion

Terpenes are a large and diverse class of organic compounds, produced by a variety of plants, particularly conifers. They are derived biosynthetically from units of isoprene (**23)** [53]. Terpenes are mostly employed in flavors and fragrances (F&F) industries. The most common sources of terpenes are turpentine oil, a subproduct of paper pulping, and citrus oil. α- and β- pinene (**24,25**) are obtained from the first and limonene (**26**) from the latter. Usually to synthesize those products via standard oil-chemistry is cheaper than extracting and purifying them from renewable sources. Common target molecules for terpenes conversion are p-cymene (**27**) or campheolenic aldehyde (**28**), all intermediates for the F&F industry. Figure 1.6 shows some examples of terpenes.

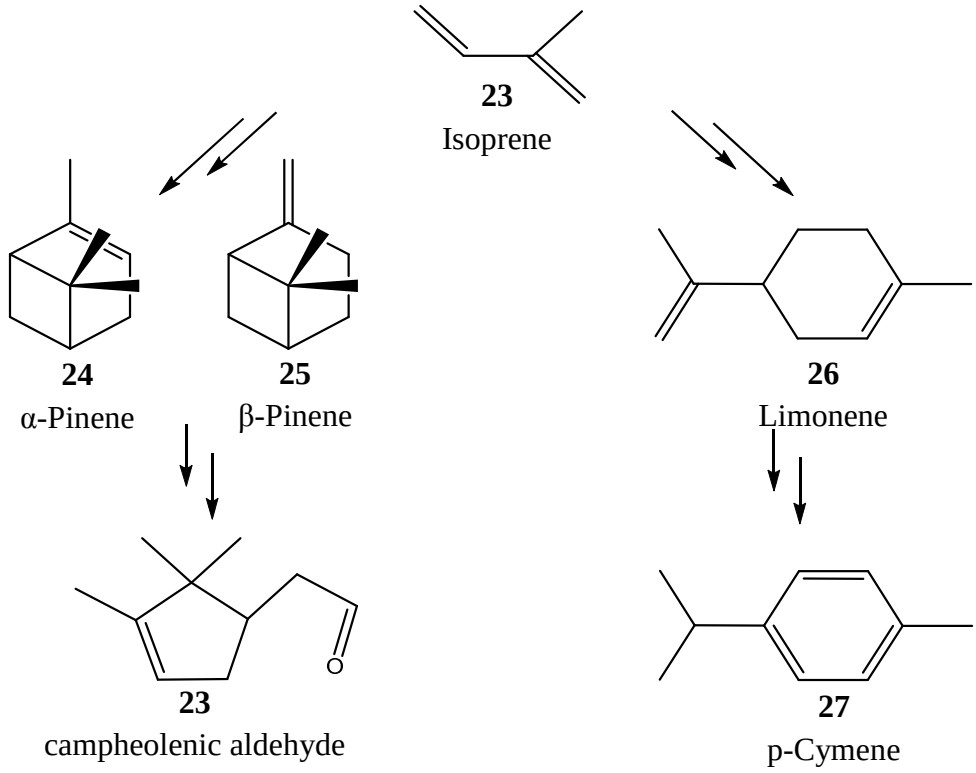


Figure 1.6 shows some of the terpenes most employed in industry as feedstock, their biological precursor (top) and some of their interesting derivatives (bottom).

Conclusions

A common feature in most of the applications is the amount of research devoted to find solid catalysts for existing processes [54]. Solid catalysis is loved by industries because it drastically reduces production costs, therefore research on solid catalysts achieve the goal of making biocompatible processes economically viable. This is not the only advantage provided. Using a solid catalyst usually means reducing the amount of solvent used or being able to separate a product by filtration instead of distillation. Of course this is the reason for the great economical advantage of solid over liquid catalysts, but what is usually forgotten is the ecological advantage. Less solvent also means less solvent leaking in the environment, smaller plants and lower risk of accidents. Less energy employed for separation means less CO_2 emission. And solid catalysts are less likely to leak out of the reactor than liquid ones, reducing the risk of environment pollution. Even though every application reported uses edible feedstock, it is possible to use wastes or non-food competitive plantation instead. Just as for biofuels, cellulose (**4**) from agricultural wastes could be used as a substitute for commonly employed carbohydrates, while waste oil could replace vegetable oil. Of course, just as for biofuels, employing wastes as feed causes low yield and production problems, linked to impurities, which need to be overcome. The way to a biobased chemical industry is still long.

1.1.3 CONCLUDING REMARKS

The advantages and disadvantages of biomass, compared to other renewable resources and compared to fossil fuels, have been discussed and some examples of applications of biomass in industry have been made. Everything will be shortly resumed here. The main advantage of biomass is being a renewable resource that produces carbon. This leads to:

1. Unique possibility, among the other renewable resources, of sustainably produce chemicals.
2. Production of fossil-like fuels, which can employ current technologies and can be stored.
3. Reduction of pollution, with CO_2 neutral fuels and biocompatible chemicals.

As a bonus, biomass resources are more evenly distributed in the world than fossil fuels, increasing energy independency for many countries.

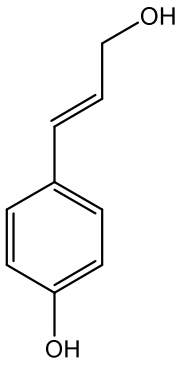
Biomass has also several disadvantages, in particular:

1. Non cost-competitive with petrol-derived products. (Just like many other renewable resource so far).
2. Chemicals could be efficiently produced only trough a brand new chemistry. Every synthesis should be renewed and reinvented.
3. Competitive with food production. Employment of wastes to avoid competition leads to several production problems.
4. Low energy production potential, at least another renewable resource will be needed.

Overall biomass conversion is a wide, complex and expanding sector, with many particular aspects that still need to be researched. Our work deals with one of those specific aspects: lignin, which will be discussed in the next chapter.

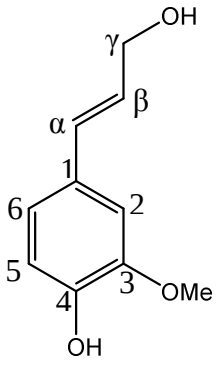
1.2 Lignin

Lignin is one of the major constituents of wood, amounting to 20-30% of wood dry matter. This makes it the second most abundant biopolymer on earth, after cellulose (**4**). Lignin covers several fundamental roles in plant biology, for example it increases the cell wall resistance to compression, which is crucial for wood structural integrity. Moreover lignin is the compound responsible for waterproofing the cell wall, which allows transport of water and nutrients in the plants vascular system. Finally, lignin plays a role in plants defense against pathogens attacks. Evidence suggests that lignin has evolved in plants during land colonization [55]. Lignin is a polymer mostly constituted by three hydroxycinamyl alcohols, which only differ in the degree of methoxylation: p–coumaryl (**28**) (MH), coniferyl (**29)** (MG) and sinapyl (**30**) (MS) alcohols. When they are linked to the polymer matrix they are usually referred to as p-hydroxyphenyl (**31**) (H), guaiacyl (**32**) (G) and sinapyl (**33**) (S) phenylpropanoid units. Lignin monomers, with carbons nomenclature are shown in Figure 1.7.



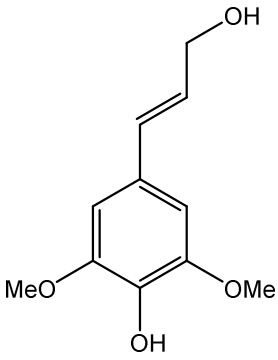
28

p-Coumaryl alcohol



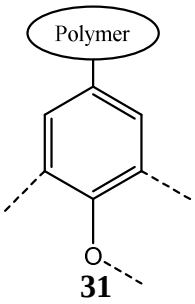
29

Coniferyl alcohol



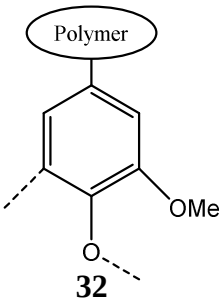
30

Sinapyl alcohol



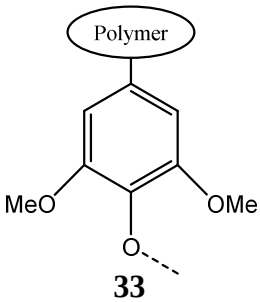
31

p-hydroxyphenyl unit



32

Guaiacyl unit



33

Sinapyl unit

Figure 1.7 shows the most common lignin monomers and repeating units, together with the most used carbons nomenclature.

Unlike other biopolymers lignin is not formed by chiral monomers and does not present optical activity. The relative amount of each component is different in each taxon but as a general rule dicotyledonous angiosperms (hardwood, such as oak, birch and poplar) are mostly composed by S and G units, with small traces of H units. Gymnosperm (softwood, such as pine and spruce) are almost exclusively composed by G units with small amounts of H units. Monocotyledons (grasses) are composed by similar levels of S and G units and relevant amount of H units. This distinction is not very accurate; many exceptions exist for each category. Differences in lignin composition are not only caused by dissimilar monomers concentration, but also by differences in structure and links between monomers. In fact, monomers couple in a seemingly random fashion in order to synthesize the lignin polymer, which means that lignin could not only be different between taxa, but also between plants of the same species. This topic is currently under debate and weather lignin is synthesized in a random or protein mediated way is one of the hottest topic in lignin research. This debate will be discussed later (p.52 section 1.3.3), for now it is sufficient to say that there is no definitive evidence for either theory. What can be taken as a fact

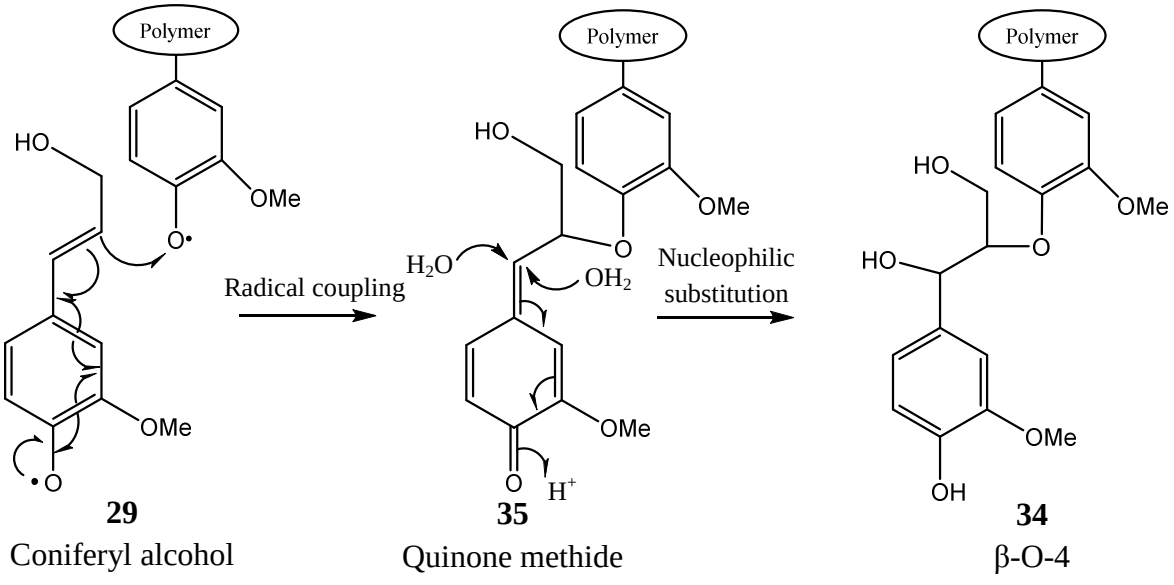
though, is that lignin has an incredibly complicated structure. Even if an ordered, protein mediated structure exists, it has not been found yet. Lignin composition is also influenced by plants age, which increases the complexity of the picture [56], but what really makes lignin structure so elaborate is the amount of possible links between constituting units. Lignin synthesis starts with radical oxidation of one of the monomers, usually oxidizing a free alcohol. The radical is stable due to the many possible resonances provided by the aromatic ring and the conjugated double bond. This radical then reacts with another radical monomer or with the growing polymer chain, the latter being by far the most likely option. As the radical electron can be localized in many sites, all these sites are then viable for the formation of the new bond, which leads to several possible inter-unit linkages.

1.2.1 LIGNIN LINKAGES

Lignin, or as someone calls it, lignins, due to the wide variety and diversity of this polymer, present certain prominent inter-unit linkages. As said before, most of the linkages derive from monomer-oligomer or oligomer-oligomer coupling reactions, while monomer-monomer couplings have a small influence, probably because of the extremely diluted reaction conditions. The name of the linkages is determined by the carbon atoms involved in the bond, according to the nomenclature shown in Figure 1.7. The most important linkages are arylglycerol *β*-aryl ether **(***β*–O–4) (**34**) and phenylcoumaran (*β*–5) (**36)**, (probably derived from monomer oligomer couplings) biphenyl (5–5') (**37**) and biphenylether (4–O–5') (**39**), (from oligomer-oligomer couplings), resinol (*β*–*β*) (**40**) and 1,2-diarylpropane (*β*–1) (**41**) couplings. A detailed description of the most important linkages is given below. Not every linkage appears in lignin with the same frequency, and not every monomer is available for every kind of linkage. For example S units (**33**) cannot form a bond in position 5, while *β* is the most reactive position and most usually connected. Some bonds are predominant in dimers formation, but they are much less present in lignin and vice versa. For example 5-5 and 4-O-5 bonds are not present in dimers, while *β*–*β* bonds are not likely found in polymers. Whether this is under simple chemical and kinetic regulation or it is protein mediated will be discussed in the appropriate section (p.52 section 1.3.3).

Arylglycerol *β*-aryl ether (*β*–O–4)

The *β*–O–4 bond (**34**) is the most abundant link in lignin and the most easily broken during pulping processes. *β*–ethers (*β*–O–4 linked units) are formed after radical reaction of the *β* position of a monomer with a free oxygen in position 4. Addition of water to the quinone methide intermediate (**35**) re-establishes the compound aromaticity and leads to the final product. Every monomer reacting at the *β* position has a quinone methide intermediate and undergoes a similar reaction. As shown in Figure 1.8 the water molecule can attack the quinone from both sides, resulting in two distinct isomers, *erythro* and *threo*. This nomenclature comes from carbohydrate chemistry and it is just one of the many existing ways to distinguish between isomers. The isomers are different in both physical and chemical properties and therefore it is relevant to understand the mechanism behind their distribution. Evidence suggests that the reaction is kinetically controlled and that thermodynamics plays a secondary role. Guaiacyl *β*–ethers are present in lignin with a 50:50 erythro/threo distribution, consistent with thermodynamic equilibrium, but in Syringyl *β*–ethers the ratio is 75:25 where thermodynamic distribution should be 55:45 [57,58]. Similar data are gathered from *β*-*β* linkages, of which only one isomer is usually found instead of the 1:1 ratio expected from thermodynamics [59].

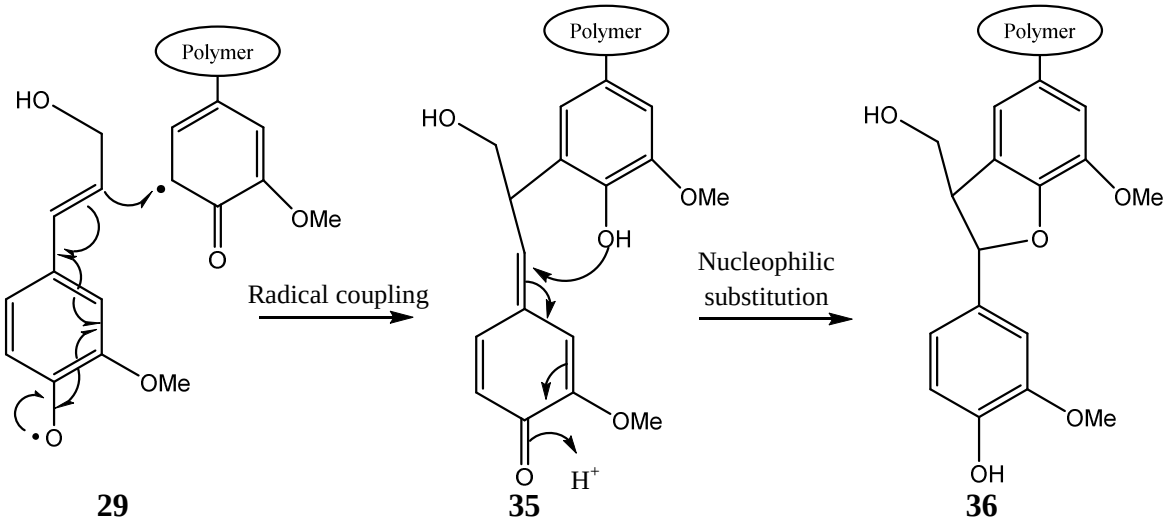


29 Coniferyl alcohol **35** Quinone methide **34** β-O-4

Figure 1.8 shows the reaction mechanism for the formation of the β-O-4 linkage. After radical coupling and quinone methide intermediate formation, a water molecule attacks the α position and restore aromaticity.

Phenylcoumaran (*β*–5)

Phenylcoumaran (**36**) is another major linkage in lignin. As previously discussed, the mechanism for its formation includes radical reaction between position *β* of the first monomer and position 5 of the second, to produce the quinone methide intermediate (**35**). As opposed to the *β*–O–4 bond (**34**), in this reaction water does not attack the quinone methide. The α position of the first monomer is attacked by the free phenol of the second, which restores aromaticity and forms the *β*–5 bond. Reaction mechanism is shown in Figure 1.9.



29 Coniferyl alcohol

35 Quinone methide

36 *β*-5

Figure 1.9 shows the reaction mechanism for the formation of the β-5 linkage. In this reaction, after the formation of the quinone methide intermediate, the aromaticity is restored by nucleophilic attack of the free alcohol on position 4.

Dibenzodioxocin ring and 5-5' bond

The 5-5' (**37**) bonds were considered to constitute a relevant part of lignin linkages, but this notion has been drastically changed about 20 years ago [60]. As shown in Figure 1.10, after the 5-5' bond has been formed, the growing chain will be further oxidized to form a radical in the 4-O position. This can react with a radical monomer in the most reactive *β* position to form a *β*–O–4 linkage (**34**), as discussed above. Instead of water addiction on the quinone methide intermediate, an intramolecular reaction, with the free phenol present in the second unit involved in the 5-5' bond, is more likely to happen. This creates an 8 member ring which includes the two original units from the 5-5' bond and a third

monomer, bonded in *α* and *β* positions. This unit is named dibenzodioxocin (**38)** ring. Most of the 5-5' bonds in lignin are now considered to be part of a dibenxodioxocin ring. This unit, together with 4-O-5 (**39**), is responsible for branching points in lignin, which would otherwise be linear. Both 5-5' and 4-O-5' bonds are shown in Figure 1.11, along with other lignin linkages.

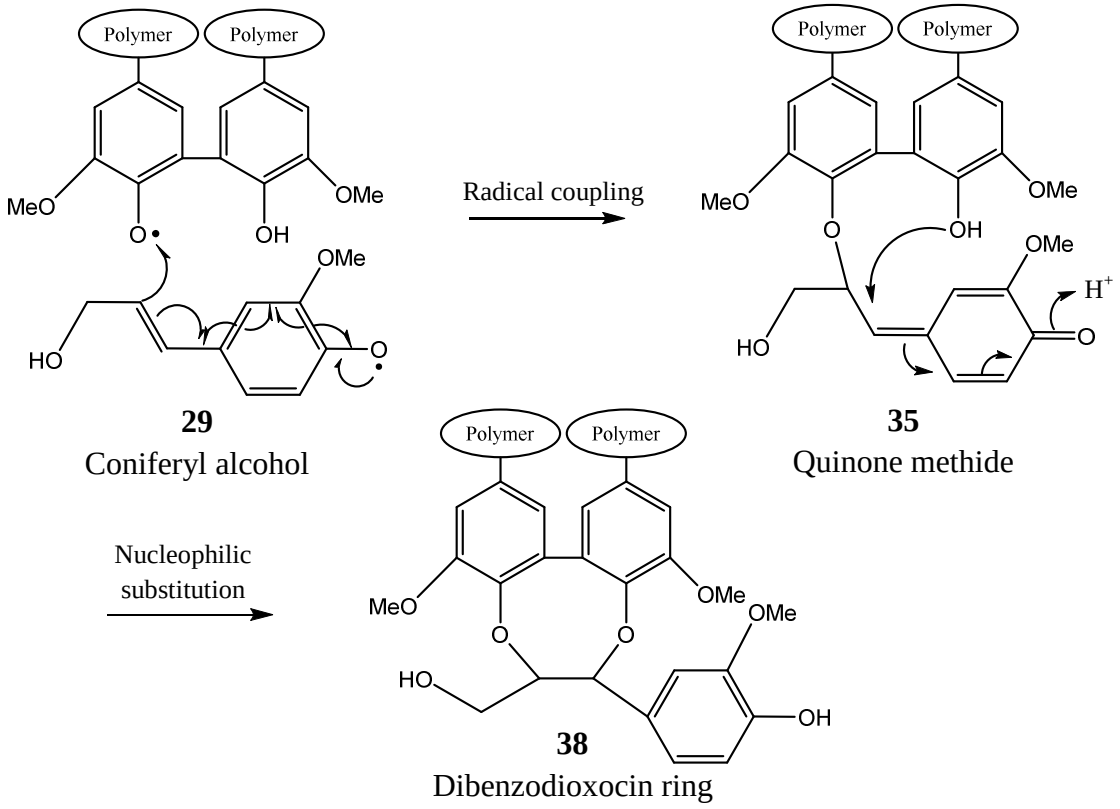


Figure 1.10 Shows the formation of a β-O-4 bond on an already formed 5-5' linkage and the following closure of the dibenzodioxocin ring. The ring closure proceeds by free alcohol attach on the quinone methide, to restore aromaticity.

Resinol (*β–β*), 1,2-diarylpropane (*β*–1) and other bonds.

Resinol (*β–β*) (**40**) bonds cover a minor amount of lignin linkages. They were initially thought to originate from two monomers coupling in the most favorable position for both, to form the *β–β* dehydrodimer, pinoresinol. This dimer would then be the starting point of the growing polymer chain, explaining the low concentration of this bond. This theory was later disputed by degradation study. *β*–ether cleaving methods (acidolysis, thioacidolysis and others, discussed in detail pp. 39-40) revealed only traces amount of pinoresinol and early work evidence of *β–β* presence was found to be wrong [61-63]. Further investigation of thioacidolysis trimers showed that most *β–β* units are linked with 4-O-5' (**39**) bonds,

which would explain the low yields obtained with simple dimers analysis. The reasons for this phenomenon and its implications are still being discussed [64].

The *β*–1 (**41**) bonds are less frequently discussed, compared to others. Their synthesis mechanism is more complicated than the ones proposed above, but always based on similar reactions. A proper description of the mechanism can be found in several reviews [65-66]. *β*–1 bonds are usually attached to preexisting *β*–O–4 units (**34**), which might explain why they are so prominently detected in degradation analysis[61,63].

For what concerns 4-O-5’ and 5-5’ (**37**) linkages, mentioned above, their synthesis mechanism is a simple radical coupling between the interested positions, followed by rearrangement to reestablish the aromaticity of the rings. Figure 1.11 shows these four linkages, without the synthetic mechanism.

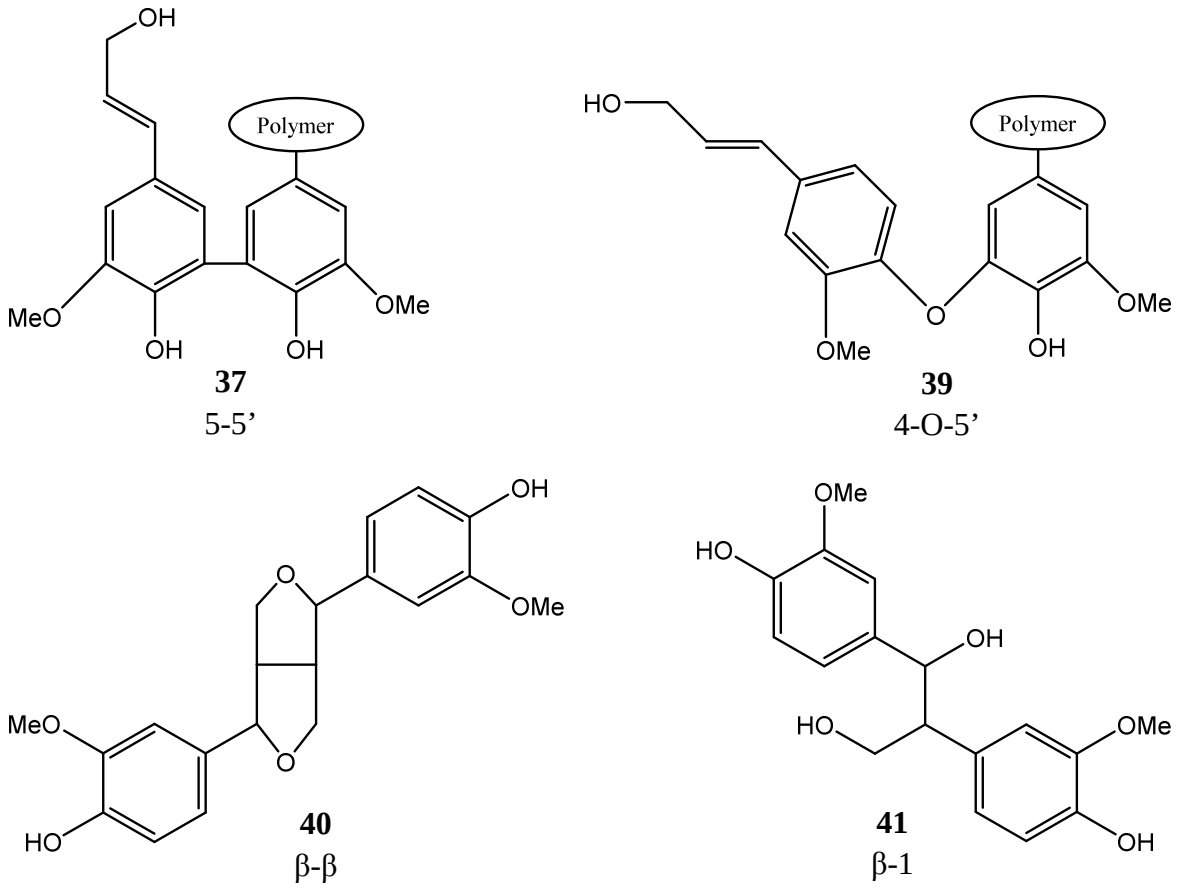
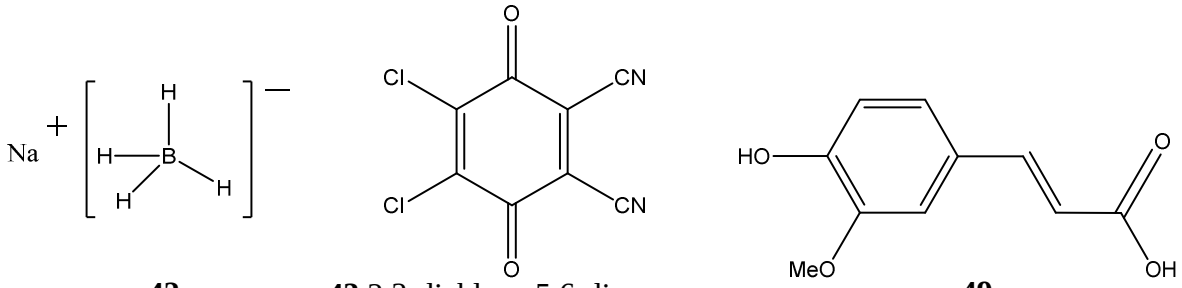


Figure 1.11 shows β–β, β–1, 4-O-5’ and 5-5’ linkages. The reaction mechanism is not shown.

Cross-links

Quite some time has been devoted to the identification and description of the most common linkages between lignin monomers. It is now time to focus on the relation between lignin and other cell wall elements, in particular polysaccharides. There are several kind of bonds between lignin and polysaccharides, the first is an ester bond between a glucuronic acid (**15)**, present in hemicellulose (**5**), and any free hydroxyl group present on lignin surface, mostly in α and γ positions. Evidence for the existence of these links is provided by borohydride (**42**) reduction and 2,3-dichloro-5,6-dicyano-p-benzoquinone (**43)** oxidation studies [67]. Esters in α could also be formed via quinone methide intermediate (**35**), after direct attack of glucuronic acid. Lignin can also be linked to hemicellulose via ether links. Most likely Glucose (**6**) and Mannose (**9**) units are responsible for those links, through reaction of a hydroxyl group with a quinone methide on lignin surface [67]. The third kind of link is provided by hydroxycinnamic acids. They are known to produce ester bonds with polysaccharides and ether or ester bonds with lignin. Ferulic acid (**49**)(fig. 1.14), being bifunctional, is capable of joining lignin and hemicellulose together by bonding a polymer on each side, creating an ester-ether bridge. Several bonds on both sides are possible. Most likely ferulic acid incorporated in polysaccharides will react either at the α position, via quinone methide reaction, or will be incorporated in lignin radical oxidation reaction and form a *β*–ether [68,69]. Every cross link discussed above is between hemicellulose and lignin. There is no solid evidence of links between lignin and cellulose (**4**) or lignin and proteins at present, even if current research and beliefs point in that direction. The mentioned molecules are reported in Figure 1.12



42 Sodium Borohydride

43 2,3-dichloro-5,6-dicyano-p-benzoquinone

49 Ferulic acid

Figure 1.12 Shows 2,3-dichloro-5,6-dicyano-p-benzoquinone, sodium borohydride and ferulic acid. The first two are employed for the anlaysis of lignin cross links. Ferulic acid is involved in cross-links formation . Due to its bifunctionality, it can bond with both polysaccharides and lignin. The two ends of the molecule can form ether or ester bonds with other alcohols or quinone methides. Ferulic acid can be linked to lignin by radical oxidation and coupling.

1.2.2 LIGNIFICATION

Xylem is one of the two types of transport tissue in vascular plants and its main function is the transport of water and nutrients. Lignin deposition takes place at the end of xylem cells differentiation process, during secondary thickening of the cell wall. Cell wall is composed by three layers: inner (S1) middle (S2) and outer (S3) layers. Lignin deposition always occurs after carbohydrates deposition and it is strictly regulated both temporally and spatially. Deposition starts in the cell corner, in the middle lamella and in the primary wall, once the carbohydrates deposit in S2, lignin starts growing in the secondary wall. Lignin penetrates the porous carbohydrate matrix, creating bonds and hardening the structure of the cell wall, making it capable of resisting weight and tension [70]. Lignin concentration is higher in the cell corners and in the middle lamella, however the highest amount of lignin is found in the secondary wall, because it includes bigger parts of the cell wall. Environmental conditions can modify lignifications, for example tension wood in angiosperms has a higher crystalline cellulose (**4**) content and lower cellulose concentration, while compression wood in gymnosperms is just the opposite. Monolignols concentration and distribution is regulated as well. Lignification begins with high concentration of H units (**31**), followed by G (**32**) and later on S units (**33**). Monomers distribution is not only temporally regulated, but also spatially. Different parts of the plant are composed by different monomers concentration, which is also valid for compression wood in gymnosperms [71].

Hypothesis and theories

A great deal about how lignification happens is already known, what is not still properly understood is how everything is controlled and what the implications are. For example, the influence of carbohydrates on lignin growth is well documented by several studies, both on in vivo lignin and on in vitro dehydrogenation polymer (DHP). Cellulose (**4**) is necessary for lignin localization, without cellulose, lignin spreads across the cellule. Moreover, evidence suggests that cellulose presence is also relevant for lignin orientation [72,73]. Yet, it is still unknown what kind of bonds or interactions are formed between lignin and cellulose, and how its control over lignin actually happens.

It is known form electron microscopy that 3D lignin growth is organized, that in middle lamella the polymer grows in spherical aggregates, while in secondary wall is more stretched and oriented, but again the reasons or causes behind this phenomenon remain mysterious [74]. It has been explained that each kind of monolignol is deposited at specific times and in different parts of the plant, and of course such a precise organization needs some sort of control mechanism [56]. The most likely hypothesis is that monolignol diffusion is controlled by the biosynthesis reaction rates, and by the selectivity of each enzyme involved in monolignol synthesis, but no definitive evidence of this fact exists.

Moreover, it is uncertain how monolignols are actually transported to the cell wall from cytoplasm after their synthesis, another factor that could help understanding how their distribution is controlled. The traditional theory is that coniferyl alcohol (**29**) is transported as glucoside into the cell wall and then liberated. This theory originated from the presence of UDP-G1c: coniferyl alcohol 4-O-glucosyltransferase in cytoplasm and of a coniferin-specific 4-O-glucosidase in the cell wall. Those two enzymes are supposedly responsible for the formation and braking of the glucoside, respectively. The activity of both enzymes does not significantly decrease with sinapyl (**30**) alcohol as a substrate. Therefore coniferyl alcohol (as well as the other monomers) is thought to be converted to coniferin (coniferyl alcohol glucoside) in cytoplasm, diffused into the cell wall and then reconverted to coniferyl alcohol by the 4-O-glucosidase [75]. This theory cannot explain what happens to the glucose (**6**) liberated in the cell wall after glucoside scission. There is no evidence of free glucose in the cell wall and usually carbohydrates deposition is completed once lignin deposition starts [65]. Therefore either this theory is wrong or it is incomplete, but no other solutions or hypothesis have been presented so far.

The last doubt about lignifications concerns nucleation sites. How and where lignin starts to grow? There are three possible candidates for this role. The least likely hypothesis is that

the enzymes responsible for polymerization are trapped in the cell wall, but empiric evidence disagrees with this hypothesis [76]. Arrays of dirigent sites, constituted by proline rich proteins, are also thought to be contenders for the role of nucleation sites. The array of proteins would start the polymerization and control the orientation of each monomer in the growing chain [77]. Finally, the last option is to consider ferulic acid (**49**) responsible for binding the first lignin monomers to the polysaccharide chain. As explained before ferulic acid is bifunctional and while trapped in the carbohydrate chain could participate to lignin polymerization and act as a starting site [78]. There is no compelling evidence for either of those two last options, nucleation sites are still an open debate in lignin chemistry.

Peroxidases and Laccases

Another topic that is still partially unknown in lignification is the role of peroxidases and laccases, the enzymes supposedly responsible for monolignols radical oxidation. Peroxidases are a family of glycoproteins, containing a heme group and usually monomeric. *In vitro* experiences prove that peroxidases are able to radically oxidize cinnamyl alcohols and other phenolics in presence of H_2O_2 [65]. Laccases on the other hand, are blue metallo-proteins, with 4 Copper atoms in the catalytic center. Laccases employ molecular oxygen as a reactant to oxidize phenolic substrates. It has not been confirmed by unquestionable experimental proof which enzyme is involved in lignification, so far circumstantial evidence suggests that both might. In any case, the role of the enzyme is to oxidize both the monomer and the growing polymer, before actual polymerization can happen. The most credited hypothesis is that the enzyme does not directly oxidize the growing polymer chain; instead a free electron is transported to the polymer and exchanged via radical transfer by small oxidized molecules, like monolignols. There are several reasons why, even after 60 and more years of research the role of these enzymes is still uncertain. First of all, the wide amount of isoenzymes in different plants, all able to oxidize monolignols but with different specificity and reactivity, makes generalization difficult [79]. In addition, the overall complexity of the lignifications process creates problems with enzyme sub cellular location and temporal correlation. For what concerns peroxidases, endogenous H_2O_2 has been detected in lignifying tissues and addition of an H_2O_2 scavenger caused lignin content to decrease [80]. Genetic upregulation of peroxidase activity increased lignin content, but its downregulation did not have any remarkable effect [81]. As stated before, involvement of peroxidases in lignifications is plausible, but not certain. Colocalization of H_2O_2 in lignifying tissues could be related to

other processes and the increase of lignin content after upregulation could only be a side effect. Laccases presence have been found in lignifying tissues, tightly bound to the cell wall, and oxidase activity different from peroxidases have been detected during lignifications [82]. It is possible that both enzymes are involved in lignifications, maybe at different times. Laccases could be responsible for the beginning of polymerization reaction and monolignol-monolignol coupling, while peroxidases would intervene later on, during the polymer chain growth. Those are only speculations and more concrete evidence is necessary.

1.2.3 APPLICATIONS

After a theoretical treatment of lignin, it is necessary to describe what is the real influence of this biopolymer in the world, which are the possible applications and how is it relevant for the industrial production. For many applications, lignin is usually considered a problem and obstacle to be overcome. The reason is that most applications for wood require cellulose (**4**), and lignin is just an impurity that needs to be eliminated. An obvious example is paper industry, where cellulose is required to be as pure as possible to obtain white paper.

Paper industry

Cellulose (**4**) is required to produce paper, but what is more relevant for our topic, is that lignin is considered an impurity with adverse effects and therefore several strategies for its removal have been designed. The process of separation of polysaccharides from lignin is called pulping and it is accomplished in several ways. The most relevant is Kraft or sulfate pulping, which covers about 70% of all chemical pulping production. During Kraft pulping wood chips are cooked with the so called white liquor, a solution of NaOH and NaS_2. During cooking the sulfite and the hydroxyl base react with lignin, mainly breaking the α- or β- ether bonds. Most reactions take place via nucleophilic addiction of the base to a quinone methide (**35**) intermediate. The ether bond is then cleaved by internal bonds displacement thanks to the participation of the introduced neighboring group, as shown in Figure 1.13.

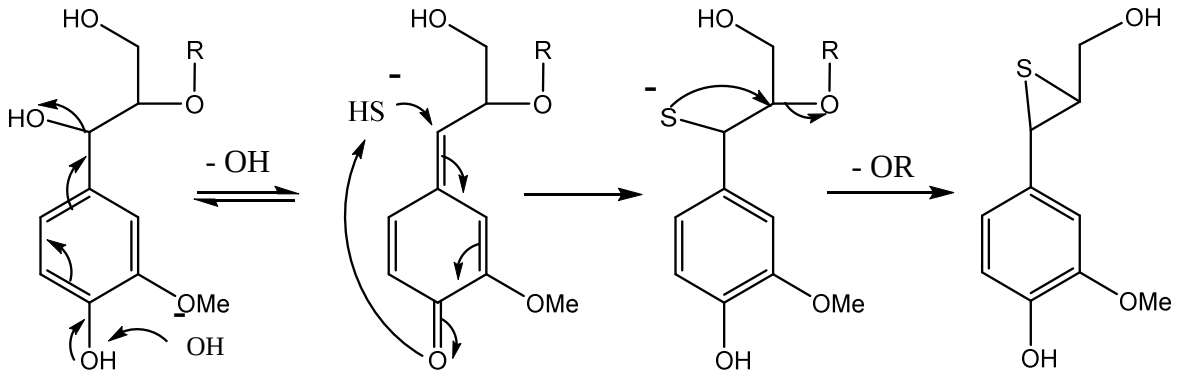


Figure 1.13 shows the mechanism of one of the most common reactions in paper pulping. The pulping base reacts with the quinone methide intermediate and then breaks the ether bond by internal bonds displacement.

Other side reactions are possible, but a specific study of pulping is beyond the purpose of this work. A complete analysis can be found in specific reviews [83]. It is enough to say that during pulping the easily breakable ether bonds in lignin are cleaved, while the stronger C-C bonds are retained. As a result, about 95% of lignin becomes soluble and it is then easily separated from the insoluble cellulose bulk. The aqueous phase now contains exhausted chemicals, the residual lignin and all the aliphatic acids produced by pulping reactions, together with every chemical derived from other lignin components (extractives etc…). This aqueous phase, made dark by the molecules in solution, takes the name of black liquor and it is combusted for energy production and chemicals recovery. After the pulping step, cellulose needs to be bleached before white paper can be obtained.

Other pulping processes include sulfite pulping, where pH can be regulated to obtain different results, anthraquinone pulping and soda pulping.

Another option scarcely considered is organosolvent pulping. It is based on dissolving lignin in organic solvents such as phenol, cresol, methanol (**1**) or others with the help of acid or basic catalysis. Finally, some mechanical pulping processes are possible, but the yield and the separation degree are not adequate for chemical industry.

Other applications hindered by lignin

As widely discussed, (pp. 10-22 sections 1.1.1, 1.1.2) when lignocellulosic materials are employed for biomass conversion in place of high sugar containing feedstock, it is necessary to use pure cellulose (**4**) as feed. The same pulping processes employed in paper industries can be applied in this field.

Another field that is negatively affected by lignin is cattle feeding; in fact it has been found that lignin content decrease digestibility in grasses. More specifically, this effect is determined by the presence of ferulate cross links and not directly by the lignin content, but no decisive evidence is available. The research in this field is currently pointing toward plants genetic modification, in order to reduce either the lignin content or the frequency of ferulate cross links. Of course, before even thinking of introducing genetically modified organisms in our food chain, their effects and ecological impact should be carefully studied.

Lignin applications

Despite being a hindrance for many applications, lignin can also be used to produce something useful. Few or any projects for lignin employment are currently available for industrial level production, therefore lignin is only considered as a waste sub product of paper production and it is normally burned. Being able to exploit better this huge renewable resource could be an important step toward sustainability, which is why more and more studies are focused on lignin conversion. Research on lignin applications is at a very early stage, but it is spread in many different sectors.

The most relevant process, probably the only industrial conversion of lignin, is the production of vanillin from catalytic oxidation of lignosulfonate. Purification of the reactant obtained from black liquor or other pulping byproducts is expensive and vanillin produced from traditional oil chemistry is still more economically convenient [84]. Conversion of lignin to chemicals is at a very early stage, preliminary studies are being conducted on lignin, mostly oriented toward enzymatic reactions. Different kinds of lignin are being tested in different reaction conditions to discover which products are easily generated and whether or not there is a chance to obtain useful molecules [85]. Less complicated studies are aimed toward using lignin as a material without major modification, for example as a building or insulant material. Even without a high added value these applications offer the chance to employ a waste material for something useful and could potentially make use of high amounts of lignin. In a recent study lignin was used

to synthesize a polyurethane-based geocomposite, polymeric foam injected in the ground to make it more resistant. This foam could be employed in mines, to prevent the risk of tunnel collapsing. Other than recycling a waste product, this application offers another advantage. Being derived from biomass this polymer is biodegradable and much more eco-friendly than a regular polymer, a relevant feature for something that has to be injected in the ground [86]. Several other possible uses of lignin are being investigated, but any real introduction of lignin as an industrial feedstock is still far away.

1.2.4 LIGNIN ANALYSIS

From what said so far, it is easy to reckon that lignin is a very complicated and hard to analyze molecule. In fact, even after more than a hundred years of research there are still areas widely unknown and fiercely debated. The reason for this is not only in the complexity of the molecule itself, but also in the problems connected to lignin analysis. These problems and the most common analytic tools will be described in this chapter. Lignin is a big heteropolymer; composed by several monomers linked in many possible ways and this obviously results in a complicated analysis with poor results. Aside from few exceptions, no matter which analytical method is employed, big and complicated molecules result in broad and overlapping signals, lower precision and an unclear analysis overall. But if size and a complex structure were the only problems, much more about lignin would be known, by now. Huge molecules such as enzymes and proteins, far bigger than lignin, can have their structure completely solved, along with the 3D disposition of every atom. What is really holding back lignin research, are the problem related to lignin purification, the impossibility, so far, of analyzing a pure and unmodified part of the polymer. In general there are two ways to achieve such a goal, either in vivo analysis or a working purification routine. In vivo analysis of lignin is complicated by the presence of every component of the cell wall. Cellulose (**4**) and hemicellulose (**5**), proteins and everything else cover or smear every signal from lignin, making a proper structural analysis almost impossible and yielding incomplete results. On the other hand there is no purification procedure for lignin that does not modify its structure or its bonds, making any analysis that follows only partially correct. Of course even partial results are better than nothing and that is where our knowledge of lignin comes from, but these problems still stand in the way of solving lignin structure. Lignin extraction and purification methods will be shortly reviewed now, followed by the currently most employed analysis.

Extraction methods

As opposed to pulping, where the purpose is to purify cellulose (**4)** from lignin, the most common way to obtain pure lignin without carbohydrate impurities are discussed here.

There are two main procedures to extract lignin nowadays, Klason's and Björkman's methods, each with its own slight modifications. Klason lignin is obtained by removal of carbohydrates from wood, through hydrolysis reaction in acidic media. Carbohydrates are dissolved in water and lignin remains as the insoluble residue. Strong acidic conditions are required for this reaction to happen, such as sulfuric acid, 40% hydrochloric acid or other strong acid mixtures [87,88]. This method is usually employed to estimate the amount of lignin present in a sample, but purified lignin is obtained only after reaction in very harsh conditions. Many chemical reactions are expected to happen, in particular condensations, which modify the structure of the polymer. Björkman's lignin or milled wood lignin (MWL) is what is currently employed as a sample for analysis. When a suspension of wood in toluene is placed in a vibratory mill with a 9:1 dioxane/water mixture, lignin becomes partially extractable. The extraction yield is around 30%, but a carbohydrate impurity is also present, around 0.1%, depending on the milling duration and other parameters [89,90]. Purity can be increased with longer milling times and digestion with glycosidase or other enzymes. Even if this procedure is much less invasive than Klason's there are still some modifications occurring in lignin, which need to be taken into account. The milling breaks some of the weakest bonds in lignin, lowering the molecular weight (Mw). Since the bonds broken are mostly ethers, as a result MWL also has a higher amount of free hydroxyl groups than in vivo lignin [91]. The final drawback that needs to be considered is the low yield of the extraction. No matter how precise or powerful the analysis, more than two thirds of the starting sample will be lost in the extraction, which is a serious obstacle to quantitative analysis.

Analysis methods

Over the years several ways to analyze lignin have been designed and optimized, each with advantages and drawbacks. They can be divided in wet chemistry methods and instrumental methods. Wet chemistry methods are usually chemical degradation studies, where lignin is reacted is some way and the products analyzed [92]. According to the products and the kind of reaction performed, some information about the starting content can be gathered. This kind of analysis belongs to the past because it is slow, destructive and hard to perform, sometimes involving unsafe or undesired chemicals. Wet chemistry

analysis are less and less employed in research laboratories, but they are sometimes more precise and more reliable than modern technology. The most relevant wet chemistry procedures will be discussed, in particular acidolysis, thioacidolysis, and derivatization followed by reductive cleavage (DFRC). For a deeper insight in wet chemistry, which made the history of lignin chemistry, we suggest Adler's review [93]. Instrumental methods are quicker and easier to perform, usually more sensitive and non invasive. They include spectroscopic methods such as UV, IR or NMR spectroscopy and other powerful analytical tools like chromatography, mass spectrometry and others. Some time will be devoted to the most relevant of the instrumental analysis. It is necessary to remember that, even if some techniques are extremely powerful, their accuracy is limited by the starting sample limitations, as previously discussed.

Acidolysis

Acidolysis is obtained by refluxing a lignin sample in dioxane-water (9:1) with 0.2M HCl. After refluxing for 4h, the effect of acidolysis on MWL is the complete breaking of the β-O-4 (**34**) bond [94]. Cleavage of the β-ether bond produces a light fraction of monomers and dimers and a heavier fraction containing oligomers. In the past, this technique was applied to arylglycerol-β-aryl ether model compounds (β-O-4 model compound) and the outcome compared to what was obtained from a Björkman lignin sample. As the two results matched, evidence for β-O-4 bonds in lignin was found. Today, this technique can give more useful results if the heavier fractions are analyzed. As the β-O-4 is the only bond broken by acidolysis, every dimer will contain one of the other possible bonds. Each dimer needs to be purified and its structure solved. Since sometimes the reaction can considerably modify the nature of the bond, each dimer needs to be studied and the original bond identified. Once all this has been accomplished, it is possible to quantitatively analyze the distribution of the different structures in lignin, simply by counting how many dimers are present per each kind of bond. In other words acidolysis allows quantification of interunit linkages in lignin [95].

When syringyl (**33**) units are present, for example in hardwoods, the work is harder because both syringyl and guaiacyl (**32**) dimers need to be identified, but the outcome of the analysis is not affected. Despite what one might think this method is not accurate for quantitation of syrigyl and guaiacyl amounts. Monomers analysis from a birch sample yielded a higher amount of syringyl units (≈3:5 guaiacyl/syringyl units ratio), despite the fact that the S / G ratio in birch is 1:1. This is caused by the fact that monomers are

obtained only when a unit is linked on both sides by a β-O-4 bond, which is much more likely to happen for S units. In fact G units can be linked to other residues by 5-5 (**37**) and β-5 bonds, which are not possibly formed in S units. In acidolysis only dimers are used for the quantitation, which leaves a big amount of sample unknown, making the quantitation less reliable [96].

Thioacidolysis

Thioacidolysis was introduced by Nimz in the 70's [97]; this procedure accomplishes the same result as acidoolysis, with higher yields. Cleavage of the β-O-4 bond (**34**) is almost complete, leading to a 91% yield of fractions from monomer to tetramer in beech wood and 77% in spruce. Lignin is reacted with thioacetic acid CH_3COSH and boron trifluoride BF_3, followed by saponification with 2M NaOH at 60°. The final step is reaction with Raney nickel catalyst to eliminate sulfur. The higher yield allows a more reliable quantitation of lignin bonds distribution, with a procedure similar to acidolysis [98].

Derivatization followed by reductive cleavage (DFRC)

DFRC is the newest of the wet chemistry analysis and as for the other two above, the purpose of this analysis is to provide a simple, high yield and selective cleavage of the β-O-4 bond (**34**). Being the most abundant linkage in lignin, cleavage of β-ethers provides the most structural information, which is why so many analytical methods are focused on it. Reaction of lignin with AcBr leads to α-ether cleavage and formation of α-bromo derivatives. Addition of powdered Zinc catalyzes reductive elimination of Br and of β-ethers, and produces alkenes. This method was applied on lignin with yields higher than 92% and high selectivity for α-and β- ether bonds. This method is slightly better than thioacidolysis from a yield and selectivity point of view, but what makes it really better are the lowest number of reaction steps and the lack of smell from Sulfur reagents [99].

Instrumental analysis

It would take too long to discuss each instrumental analysis in detail (and it would be probably useless). Due to the wide range of application, instrumental analysis, and spectroscopy in particular, is widely spread in every field of chemistry. The main feature of each technique are well known and too complicated to be discussed here. Some specific textbooks are advised to those who want a deeper understanding of these techniques [100,101]. Here it will be just shown how modern analysis tools have influenced lignin research.

Microscopy (electron and UV) is routinely employed and usually substitutes histological analysis. Individuation of tension and compression wood, topochemical mapping of extractives and lignin, study of lignin orientation are some of the results that can be accomplished faster and better with such techniques [102-104]. Fluorescence and Raman imaging are commonly employed for compression wood studies, involving quantitation and identification, once again faster than any histological or chemical method [105,106]. Raman spectroscopy was used with good result to identify long range order in lignin, on the other hand IR spectroscopy is common in industrial application, for example in paper industry [107,108]. UV/Vis spectroscopy usually gives good results when applied to lignin, due to the high aromaticity of the polymer [109]. Chromatography (liquid chromatography LC, gas chromatography GC, size exclusion chromatography SEC) played a major role in lignin fragments separation and purification, usually coupled with mass spectrometry (MS) which provides a very fast and efficient oligomers identification routine [110]. Nuclear magnetic resonance (NMR) is probably the most important of all spectroscopic techniques in lignin chemistry and deserves a separate chapter, also because it is the main analysis technique adopted in this study.

Nuclear Magnetic Resonance

The incredibly low sensitivity is the major drawback of NMR, compared to other spectroscopic techniques. In general, spectroscopy involves transition between a ground state and an excited state, separated by an energy gap. After excitation, the population of the ground state migrates to the excited state. This migration or the following return to the ground state produces a detectable signal. This signal is what is measured in each spectroscopic technique. With techniques such as UV/Vis or IR spectroscopy, the two states are separated by a relevant energy gap, which means that there is a big difference in population between the two states. When the fist state is excited to the second, there is an

important change in population, which produces an easily detectable signal and therefore a good sensitivity. On the other hand, NMR ground and excited state are extremely close in energy, which means that that the population of the two states is nearly the same. The signal produced by population change is therefore almost undetectable, which explains why NMR sensitivity is several order of magnitude lower that other spectroscopic techniques. NMR provides incredible advantages, which easily overcome the drawback of low sensitivity. The low energy involved in the transition, other than reducing NMR sensitivity, also assures that the molecule is not modified during analysis. The amount of energy involved is in fact so small that physical or chemical modification of the molecule is impossible, allowing analysis of an unperturbed state. Moreover, NMR provides unique structural information, far more detailed than any other analysis. A molecule structure can be completely solved by NMR, together with its 3D structure, a result that can be matched only by X-ray crystallography. The aim of this paragraph is just to give a short introduction to NMR potential. NMR theory is much better explained in several books [111].

For what concerns lignin, its polymeric nature always posed some problems for NMR. Peaks overlapping and low resolution always put a limit to the amount of information that can be gathered from a spectrum, but big steps have been made to overcome this problem. More and more powerful magnet, from the 60 MHz magnets in the 70's to 1000 MHz today, provide an incredibly enhanced resolution, but the most significant step was the introduction of ^{13}C NMR, and 2D and 3D NMR experiments later on [66]. Application of experiments other than proton provides a huge amount of information about bonds and structures, not to talk about the otherwise impossible resolution that can be achieved. Hundreds of 2D NMR experiments exist today, with a variety of characteristics. HSQC correlates a carbon atom to its attached protons, NOESY provides tridimensional information, and TOCSY individuates proton systems and so on [112]. With the help of this powerful tool many problems have been solved. Assignment and quantitation of *erythro* and *threo* isomers [113,114], bonds quantitation [115], lignin amount evaluationss, all of those things and others are made much easier or even possible at all, with the help of this instrument [116]. New links and new minor monomers have been discovered [117], moreover the technology and operators proficiency is increasing so much that even whole cell wall analysis are being accomplished [118-120]. NMR is a key to lignin understanding, improvements in NMR technology have led to major breakthroughs in lignin comprehension.

1.2.5 CONCLUDING REMARKS

Lignin is a very complicated biopolymer; it is composed by three major monomers linked in several different ways, which makes it an exception among biopolymers. Each plant species presents its own kind of lignin, with differences in monomers and linkages distribution, which are also affected by environmental conditions and plant age. The complexity of lignin has only been superficially touched in this chapter and the next will not go much further. For a more detailed treatment of the subject we suggest the famous book on lignin by Sarkanen [121]. Lignin, despite being the second most abundant biopolymer on earth, is underexploited and only a few useful applications are currently viable. In fact, lignin is commonly viewed as an obstacle in biomass application, such as paper industry or ethanol production and it is usually just burned after separation. The reasons for lignin under exploitation are to be researched in its complexity. A complicated structure translates in a very difficult research, which turns into very low efficiency applications or no applications at all. A better knowledge of lignin features would surely help applicative research, but the very structure of lignin is adverse to an optimized employment. The great variety of linkages between monomers makes it hard or too expensive to degrade lignin to its components, which could be employed in industry. In fact, the enzymatic cleavage approach adopted for other biopolymers would not work, due to the high amount of different bonds, and the design of a specific chemical treatment per each link would hardly be cost effective.

If it was possible to put lignin to good use, that could be a relevant step toward sustainability, that is why many resources are being invested in this sector and why so many researches are aimed in that direction. In order to understand how to manipulate lignin it is first necessary to fully understand lignin structure, which is done by two main approaches: model compounds and biosynthesis study. Each approach will be individually discussed in the next chapters.

1.3 Lignin Biosynthesis

The study of lignin biosynthesis could and did provide useful information about lignin structure. What is better than studying how something is formed, to understand what it becomes? This field is undergoing an intense development, with many dark and debated areas. Many details about lignification that were left behind in the previous chapter will be now fully discussed and the most important points of view considered. Lignin biosynthesis is normally studied by perturbation of the biosynthetic pathway, followed by analysis of the results. By understanding what changes under a well defined condition, such as inhibition of a specific enzyme, it is possible to understand the role of each component of the very complicated biological synthesis pathway. Once the mechanisms will be fully understood, maybe it will be possible to modify and manipulate the enzymes, in order to produce a lignin with more favorable characteristics. The first part of this chapter will be devoted to describe the state of the art knowledge on monolignol biosynthesis, followed by discussion on the topics of lignification left behind in the previous chapter. To conclude, the possible applications of genetic modification of lignin will be reported.

1.3.1 MONOLIGNOL BIOSYNTHESIS

Figures 1.14 and 1.15 report the monolignol biosynthesis pathway. Phenylalanine (**44**) (Phe) and tyrosine (**45**) (Tyr), in the top left corner, are commonly considered to be the starting point of monolignol biosynthesis, but some research group are now investigating whether differentiation between protein and phenylpopanoids synthesis starts earlier. With simpler words, it is being researched if the precursors of Phe that will become part of proteins are different from the precursors of Phe that will become something else. The functions of each enzyme involved in lignin biosynthesis, and what researchers have accomplished on each one of them, will be described in the next paragraphs, individually. It is important to keep in mind that this set of enzymes does not produce only monolignols destined to lignification, but also plays a role in the synthesis of many other molecules. Description of the system beyond the synthesis of monolognols is beyond our purpose.

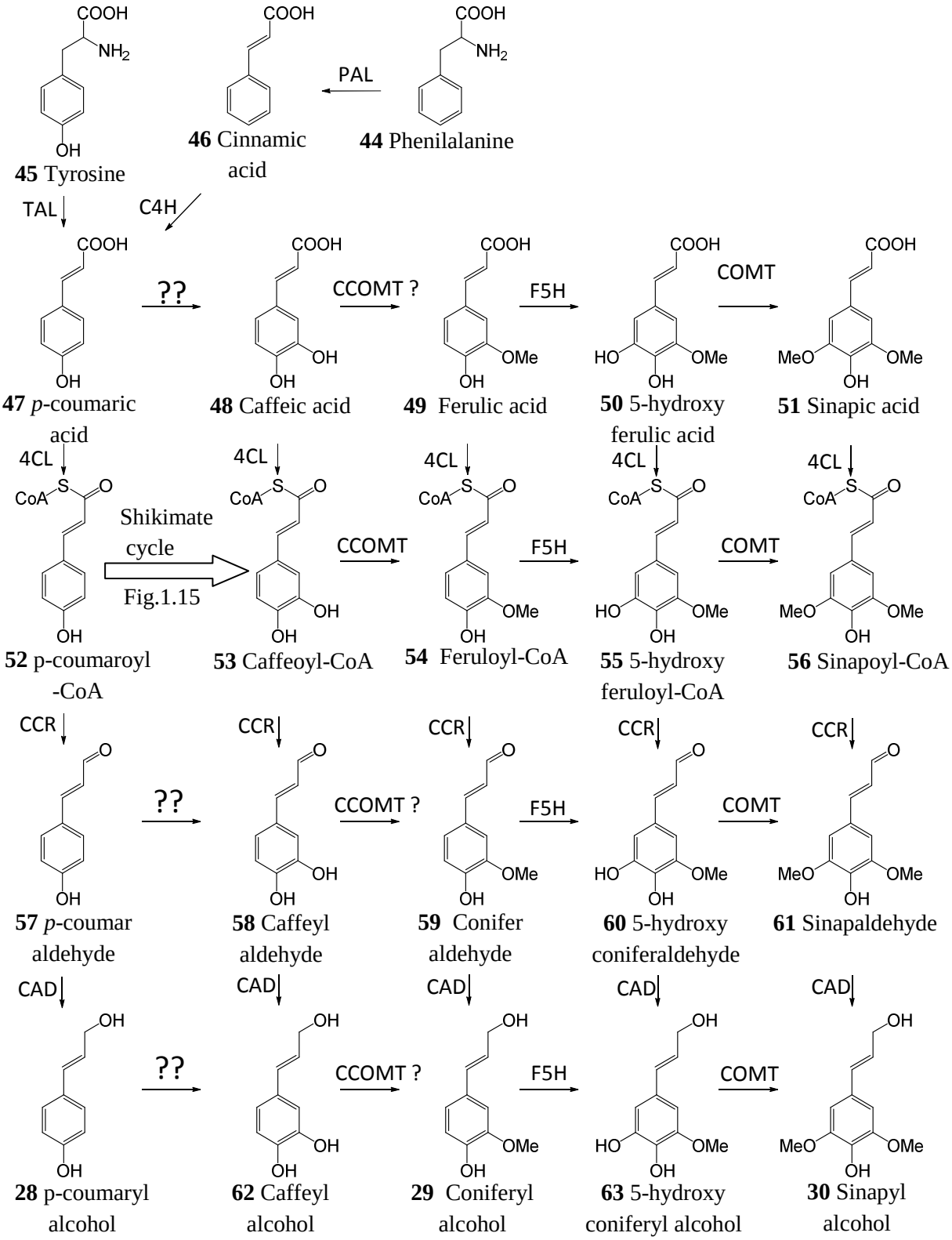


Figure 1.14 Monolignol biosynthesis pathway. Some steps "??" are still unknown. The shikimate cycle is described in fig. 1.15. PAL phenylalanine ammonia lyase, TAL tyrosine ammonia lyase, 4CH cinnamate-4-hydroxylase,COMT Caffeic acid O-Methyltransferase, F5H ferulate 5 hydroxylase, 4CL 4-Coumarate CoA ligase,CCR Cinnamoyl CoA reductases, CAD Cinnamyl alcohol dehydrogenases.

Phenylalanine and Tyrosine ammonia lyases (PAL and TAL)

As can be seen in Figure 1.14 the role of PAL and TAL is the elimination of the ammine in the two amino acids (Phe and Tyr) in order to form a double bond, which leads to formation of cinnamic (**46**) and p-Coumaric (**47**) acids. PAL is the entry point for the phenylpropanoid metabolism for most plant, only some grasses are able to convert Tyr to p-Coumaric acid. The released NH_4^+ is the recycled for the synthesis of other amino acids, which explains why lignification can continue without other sources of nitrogen [122]. The first gene encoding PAL was published in 1985 [123]. Nowadays, after *Arabidopsis* (a common model plant) genome sequencing has been completed, 4 different isoforms of PAL have been identified and are being studied.

Cytochrome P450 hydroxylases (C4H, pC3H, F5H)

Three hydroxylation steps are involved in monolignol biosynthesis. The first step is mediated by cinnamate-4-hydroxylase (C4H) and converts cinnamic acid (**46)** to *p*-coumaric acid (**47**). This is a key step for every phenylpropanoid-derived metabolites (i.e. suberins, hydroxycinnamic acids etc.). The second hydroxylation promotes insertion of an alcohol in the aromatic position 4 and converts *p*-coumaric acid derivatives (**52,57,28**) to caffeic acid (**48**) derivatives (**53,58,62**). It is mediated by *p*-coumaric acid hydroxilase (pC3H). The last step, mediated by ferulate 5 hydroxylase (F5H), inserts a hydroxyl group on carbon 5, necessary for the precursors (**51,56,61**) of sinapyl alcohol (**30**) [124].

All three of these reactions are mediated by cytochrome P450. Identification and characterization of C4H and F5H was pretty straight forward [125,126], while pC3H involved some further complications, described in the next chapter.

pC3H and hydroxycinnamoyl CoA transferases

The identity of the enzyme involved in 3 hydroxyl insertion was debated for a little longer. Doubts were present about the nature of the enzyme and its substrate. In fact, pC3H cannot convert p-coumaric acid (**47**) into caffeic acid (**48**), but p-coumaroyl shikimate (**64**) is a suitable substrate for conversion into the caffeoyl shikimate (**65**) as well as its quinate equivalent (**66**). The formation of these shikimate/quinate derivatives requires a specific enzyme, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase (HCT/HQT).The particularity of this couple of enzyme is that they show high versatility, being able to convert both coumaric and caffeic acid derivatives. Therefore HCT and HQT are able to convert *p*-cumaroyl CoA (**52**) to *p*-coumaroyl shikimate/quinate.

The latter is then converted to caffeic shikimate/quinate (**65,67**) by pC3H which in turn is reconverted to caffeoyl CoA (**53**) by the same HCT and HQT enzymes [127,128]. The shikimate/quinate pathway is shown in Figure 1.15.

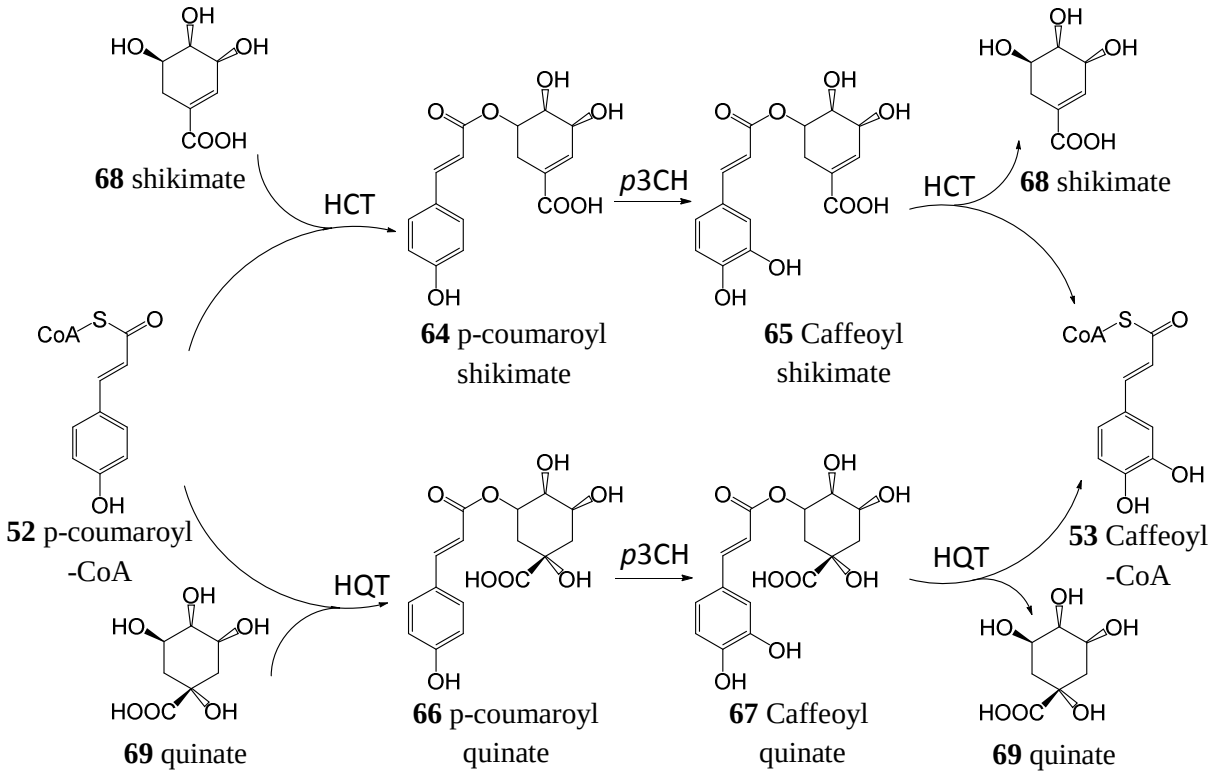


Figure 1.15 Shows the shikmate/quinate cycle. It is currently the only known pathway from coumaric to caffeic derivatives. HCT/HQT, hydroxycinnamoyl CoA:shikimate/quinate hydroxycinnamoyl transferase, p3CH, p-coumaric acid hydroxilase.

4-Coumarate CoA ligases (4CL)

The class of enzymes 4CL has a high substrate versatility. In fact, each of these enzymes is able to convert almost every hydroxycinnamic acid (**47-51**) into a CoA ester (**52-57**). The first 4CL gene was discovered in 1987 [129]. Every known 4CL has high substrate versatility, but each enzyme shows its highest activity with a different substrate. Studies have been conducted on the 4 different 4CLs present in *Arabidopsis* and each of these enzymes has a different preferred substrate. The current hypothesis is that each enzyme is specific for a different role, such as lignification, defense or flavonoids biosynthesis, and it is therefore more active with the most suitable hydroxycinnamic acid [130].

O-Methyltransferases

In the past, Caffeic acid O-Methyltransferases (COMT), were thought to be a bifunctional enzyme [131]. They were supposed to be able to convert both caffeic (**48**) acid into ferulic acid (**49**) and 5-hydroxyferulic acid (**50)** into sinapic acid (**51**), i.e. to be responsible for methylation of both hydroxyls in 5 and 3 positions. This hypothesis was based on *in vitro* study and proved to be wrong. In fact OMTs are composed by two classes of enzymes: caffeoyl CoA O-methyltransferases (CCOMT), using caffeoyl CoA (**53**) as a substrate for conversion to Feruloyl CoA (**54**) (methylation of 3 -OH), and COMT, involved in the conversion of hydroxyconiferyl derivatives (**50,55,60,63**) to sinapyl derivatives (**51,56,61**), for the production of sinapyl alcohol (**30**), as reported in Figure 1.14 [132,133]. The name COMT is still employed for historical reasons, even if the enzyme should probably be called otherwise.

Cinnamoyl CoA reductases

Just as 4CLs, also this family of enzymes has great substrate versatility. In fact Cinnamoyl CoA reductases (CCRs) are able to convert every CoA ester shown in Figure 1.14 to the corresponding aldehyde, using NADPH as a reducing agent. In particular p-coumaroyl, caffeoyl, feruloyl, 5-hydroxyferuloyl and sinapoyl CoAs (**52-56**) are converted to p-coumaryl, caffeyl, coniferyl, 5-hydroxyconiferyl and sinapyl aldehydes (**57-61**), respectively. CCRs are type B-reductases. The first CCR gene encoding was reported in 1997 [134]. Eleven (or twelve) genes are proposed as CCR but only three have been characterized so far. They display different activities and in one case, different selectivity, but the biological implications for this fact are still unknown [135].

Cinnamyl alcohol dehydrogenases

Cinnamyl alcohol dehydrogenases (CADs) are a non substrate specific family of enzyme. They are able to convert all the aforementioned aldehydes (**57-61**) into the corresponding alcohols (**28-30,62,63)** (Figure 1.14). CADs are a type A-reductases and they use NADPH as a reducing agent, extracting the 4-pro R hydrogen. Even if CADs are substrate versatile, each one presents a higher activity for a specific substrate. CAD catalytic center is based on three Zn atoms [136]. There are currently seventeen CADs reported, but some are completely different from others. The first nine show high level of similarity between each other, while the other eight are fairly different [137].The biological meaning and implication of it are still being discussed.

1.3.2 MONOLIGNOL BIOSYNTHESIS PERTURBATION

The function of each enzyme present in Figure 1.14 has been discussed in detail, now it is time to analyze the most relevant effects caused by perturbation of monolignol biosynthesis. Disruption of the ordered biosynthesis pathway will provide useful information about the regulatory aspects of monolignol synthesis and increase the general understanding of lignin formation. Once again, the topic will be discussed one enzyme at a time.

PAL and C4H

Leaving aside TAL and the minor tyrosine entry point, PAL and C4H constitute the starting point for the synthesis of every monolignol. Therefore it is not a surprise, that downregulation of those two enzymes leads to reduced lignin content [138]. On the other hand, the effects on monolignol distribution were hardly foreseen. In fact downregulation of PAL mostly reduces G units (**32**) amount, while C4H has the same effect on S units (**33**). The causes of this phenomenon are still unknown, but three hypotheses have been proposed [139]. The first is that the pathway to coniferyl alcohol (**29**) may bypass C4H, the second is that C4H is involved in more reactions other than the 4-hydroxylation of cinnamic acid (**46**) and the third is that C4H may be part of a specific metabolic channel for production of sinapyl alcohol (**30**). None of these hypotheses is supported by evidence.

4CL

The effects of 4CL downregulation are variable, depending on the tested plant. Transgenic plants usually show lower lignin content, but in certain cases an increase of hydroxycinnamic acids bound to the cell wall is observed. Most of all, the effects on S/G ratio are unpredictable [140]. In fact, tobacco shows a reduction in S units (**33**), while transgenic *Arabidopsis* displays a lower amount of G units (**32**). To make it even more uncertain, in aspen, the S/G ratio is not different from the control one [141]. These contradictory effects are probably due to that fact that 4CLs are a wide family of enzymes, each with its own substrate specificity. Therefore different effects on different transgenic plants are reasonable.

C3H

In C3H deficient Arabidopsis, a severe reduction of lignin content was measured, together with an increase in p-coumarate derivatives (**47,52,57,28**) accumulation instead of sinapoyl (**51,56,61,30**) ones. The lignin present in this transgenic line is almost exclusively composed by H units (**31**), with relevant amounts of p-coumaric esters. This suggests that most likely C3H is the only enzyme involved in the functionalization of position 3, which plays a key role in the synthesis of MG (**29**) and MS (**30**). Most likely no redundancy for this function is present in the biosynthetic pathway [142].

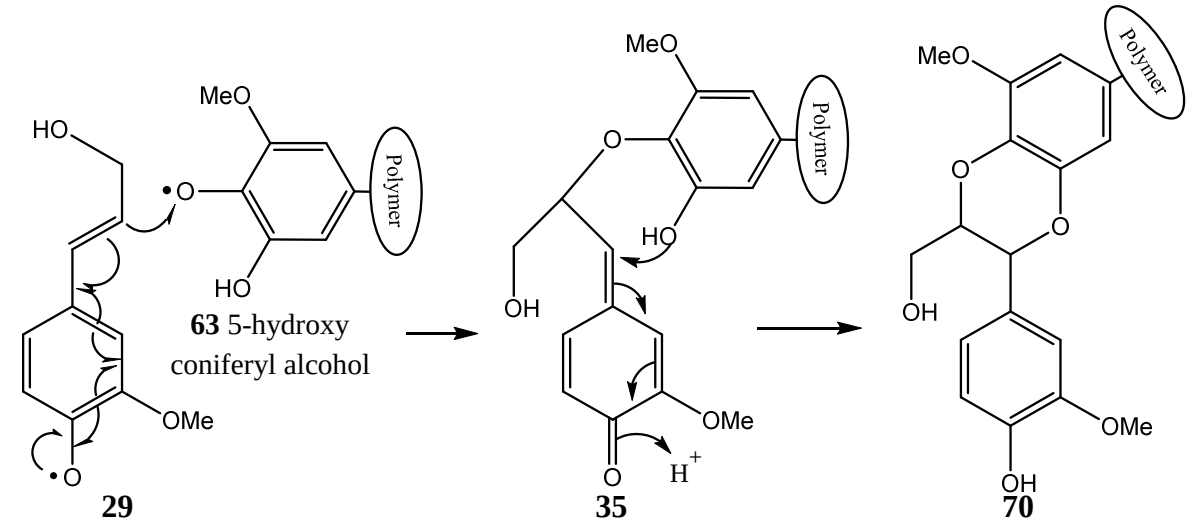
CCOMT

Downregulation of CCOMT reduces the lignin content and increases the S/G ratio, most likely because G units (**32**) production is more affected by the modification than S units (**33**) synthesis. In CCOMT deficient alfalfa MS (**30**) production is unaltered, while MG (**29**) amount decreases. This is consistent with what discussed before, i.e. that CCOMT plays a part in G units production. It also suggests that CCOMT is not necessary for MS synthesis, which implies that COMT has to be at least in part responsible for methylation of the hydroxyl group in position 3 and not specific for position 5 [143]. When CCOMT is down regulated, unusual amounts of caffeic acids derivatives (**48,53,58,62**) are detected. This is probably caused by caffeoyl CoA esters accumulation due to low CCOMT activity. Caffeoyl esters are converted to caffeic acid by thioesterases, which explains the amount of caffeic acid derivatives.

COMT

As for almost every other enzyme, reduced COMT activity decreases the amount of lignin. Downregulation of COMT leads to reduced production of MS (**30**) and accumulation of non methylated 5-hydroxyconiferyl alcohol (**63**) instead. 5-hydroxyconiferyl alcohol is then incorporated in lignin like a normal monolignol. As a result COMT deficient plants show a reduced amount of the bonds abundant in S lignin, such as β-β (**40**), and for the opposite reason an increased amount of 5-5 (**37**) bonds. Moreover the incorporated 5-hydroxyconiferyl alcohol is responsible for a new kind of linkage, a benzodioxane unit (**70**) which involves bonding from both the C_4 and C_5 hydroxyls. 5-hydroxyconiferyl alcohol can easily be incorporated in lignin reacting in β position, as any other monolignol, but the extra free alcoholic group is available for further reactions.

The mechanism for the formation of the benzodioxane unit is shown in Figure 1.16. It is interesting that after identification of this unit in transgenic plans, faint traces of the same unit have been found in normal lignin [65].



29 Coniferyl alcohol — **35** Quinone methide — **70** Benzodioxane

Figure 1.16 shows the reaction mechanism for the formation of a benzodioxane ring. The second free alcohol present on the "incomplete" monomer attacks the quinone methide intermediate to close the ring and restore aromaticity.

CCR

Reduction of CCR activity causes a decreased amount of lignin and a higher S/G ratio. Increased amount of phenolics bound to the cell wall is also registered [144]. Those results are surprising, but they are probably caused by the vast amount and different kinds of CCRs present.

F5H

Regulation of F5H enzymes has a major influence over the presence of S units (**33**) in lignin. Downregulation of the enzyme leads to lignin rich in G (**32**), not so different from softwoods. On the other hand upregulation of the same enzyme produces an S rich lignin. In some plants, when F5H is upregulated, variable amount of 5-hydroxyconiferyl alcohol (**63**) and benzodioxane (**70**) linkages are found. This happens when COMT activity is lower than F5H's [145]. F5H is most likely the only enzyme that does not affect lignin content in wood.

CAD

CAD catalyzes the final step of monolignol biosynthesis, but its downregulation barely affects lignin content. This unexpected lack of variation is explained by substitution of standard monolignols with their relative aldehydes. In fact CAD deficient lignin presents unusually high amounts of coniferaldehyde (**59**) and sinapaldehyde (**61**) employed as standard monomers [146].

1.3.3 LIGNIN BIOSYNTHESIS: COMBINATORIAL VS. CONTROLLED

It is now time to address probably the most controversial topic in lignin chemistry. It has been brought up now and then in the previous chapters, but the evidence provided by transgenic plants was needed before this topic could be properly discussed. The main subject of discussion is whether lignin is randomly assembled under simple chemical control, in a combinatorial way, or if its synthesis is instead strictly controlled by proteins and enzymes.

Before moving on, it is important to remember that no compelling evidence is present for either one of the theories, therefore both should be equally considered.

I would like to add a little personal consideration here, the reader should take it with all due precautions. It seem to me, that people have been debating over this topic for so long that they don't care so much about the truth but about being right. Papers of groups on opposed sides are factious, only partial evidence is reported and every data is twisted and presented as if it is the definitive proof, when in reality could fit one theory just as well as the other. The debate has degenerated so much that is not uncommon to find not-so-veiled insults to the opposed faction. It's totally understandable that such things happen, after a long, frustrating and seemingly never ending argument, but we should all try to restrain ourselves and remain objective. I'll try to present the facts as impartially as I can and I hope the readers will consider them in the same way, because being open minded is probably one of the most important features or a good scientist.

History and general ideas

The combinatorial, or random, approach is currently accepted by the scientific community, mostly because it has been proposed more than 50 years ago and not really challenged until recent times. Freudenberg, in the 50's, was the first to propose that lignin was a polymer formed by the random coupling of monolignol oxidized radicals. This conception is born form the high complexity of lignin, its lack of optical activity and the apparent lack of long range ordering and repetitive structures. When structures similar to lignin were found in randomly polymerized coniferyl alcohol (**29**) (DHP), the conclusion that lignin is formed by random coupling came naturally [147]. It is important to underline that the analytical tools available at Freudenberg's times are in no way comparable to today ones. This hypothesis was accepted almost without discussion in the scientific community, even if warnings about this speculative hypothesis were given [148]. Beyond the analytical difficulties that made lignin study a very complicated field, perhaps the importance of Freudenberg name played a role in this unquestioned acceptance of his theory. Over the year many claims were made about lignin and its structure, some more speculative than others, and often only partially correct. Nowadays the random theory has evolved into combinatorial and it is partially based on Occam's razor. Sustainers of this theory claim that every feature of lignin can be explained by simple kinetic and chemical control, without the necessity of involving other non necessary entities, such as enzymes. On the other hand, the opposed theory claims that lignin synthesis is somehow controlled by proteins, called dirigent proteins (DPs), which define and regulate lignin structure. Long arrays of DPs are hypothetically present in the cell wall, where they stereochemically control every coupling reaction [149]. Even if doubts over the random coupling hypothesis have always been present, this theory acquired importance and attention in the last 20 years, after discovery of dirigent proteins involved in lignans synthesis. The foundation of this theory is inductive reasoning: as every other biomolecule synthesis is very strictly regulated by enzymes, it is highly unlikely that lignin deposition is left without control. Even if no final proof exists, a number of circumstantial data have been presented over the years. Each case will be presented individually and as impartially as possible, considering both points of view. The cases are presented in no particular order.

Lignin and DHP, same or different?

DHP (dehydrogenation polymer) includes the whole series of polymeric compounds obtained by radical oxidation of one or more monolignols, *in vitro*. DHP and its importance will be adequately discussed in chapter 1.4 (p. 58). The hypothesis of random coupling was born when the same linkages were found in both lignin and DHP, and therefore they were assumed to be the same thing. As a consequence, since DHP has to be formed by random coupling, also lignin is formed in a random process. The obvious critique moved to this statement is that DHP and lignin are not the same thing and, as a matter of fact, a DHP equal to lignin has never been synthesized [146]. The key point of the question is how DHP is synthesized. In early experiments the difference between DHP and lignin was really remarkable. Just to name one, the β-O-4 bond, the most abundant in lignin, composed just a minor fraction of DHP and vice versa DHP showed great concentration of bonds barely present in lignin. Little by little, by optimizing and improving the reaction condition (slower and slower addition of monomers, presence of carbohydrates in the reaction mixture etc.) DHP became more and more similar to lignin. Supporters of the combinatorial theory take this improvement in similarity as a proof that DHP and lignin are synthesized basically in the same way and that it is possible, in principle, to synthesize lignin *in vitro* under the right conditions [150]. On the other hand, the diversity between the two is taken as a proof that proteins are involved and that lignin and DHP are substantially different by the opposed faction.

Lignans biosynthesis

Lignans are a class of compounds very similar to lignin. They are mostly monolignol dimers coupled prevalently with a β-β bond, even if oligomers and other bonds are represented. They play an important part in plant defense, and they have been employed in medicine as antiviral or for cancer chemotherapy. Unlike lignin, most of these compounds are optically active, suggesting a stereoselective coupling. A major breakthrough in this field was achieved when (+) pinoresinol was synthesized from coniferyl alcohol (**29**) using an insoluble stem residue preparation. When the proteic part of the preparation was analyzed, a laccase and a ≈50kDa protein with no oxidizing properties were obtained. This protein, later named dirigent protein (DP), catalyzes selective coupling of (+) pinoresinol. The structure of the DP and the kinetics of the reaction were then investigated. The DP was found to exist in dimeric form. Each part would trap an oxidized radical monolignol in a specific position, forcing a stereoselective reaction to form (+) pinoresinol [151]. Other

similar proteins were found later on and are currently under investigation. This was the piece of evidence that basically started the whole protein controlled theory. Since lignans and lignin are so closely related, it is natural to think that their synthesis is comparable. On the other hand, the opponents underline that even if lignans and lignin are synthesized from similar subunits, nothing proves that their synthetic mechanism is identical, also because lignin is not optically active.

New evidence from transgenic plants

The new results from transgenic plant lines, introduced a new debate topic in this already overheated argument. As discussed before (p. 49 section 1.3.2) regulation of some specific enzymes led to incorporation of incomplete monomers and new bonds in lignin structure. This happened mostly for the couple F5H-COMT and for CAD enzymes [152]. Moreover, evidence suggests that these unreacted monolignols are present also in normal lignin. The combinatorial faction takes this as the final evidence that the protein mediated theory is wrong. In fact the ability of lignin to adapt to every situation (lack of monomers, different monomers) is considered to be unlikely achieved by enzymes. The incredible variety of conditions could hardly fit in the strictly regulated and very specific series of DPs proposed. From their sides the supporters of Protein mediation question the integrity and the effective meaning of the data proposed, mostly stating that the unreacted monolignols are not really included in lignin.

Lignin oligomers profiling

Another piece of evidence in favor of the combinatorial theory comes from the analysis of lignin oligomers. LC-MS/MS profiling of both synthetic DHP and natural lignin revealed a very strict similarity between the two. Over 38 oligomers, from dimers to tetramers, have been isolated and characterized both from synthetic and natural products, suggesting that enzymatic control is not present or unnecessary in natural lignin. According to the authors, DPs are not required to explain the reaction mechanism proposed [153]. This study does not add much to the discussion on the equality or inequality between lignin and DHP, discussed above, it just proposes more evidence of their similarity.

Lignin template polymerization

A very interesting paper has been published in favor of the protein controlled hypothesis. It proposes a model calculation that explains lignin formation. According to calculations, an array of DPs regulates the formation of a first string of lignin. In this string therefore, both monomers distribution and linkages frequency are strictly controlled. This first string then works as template for the second, which grows in the opposite sense and has therefore opposed chirality, explaining why lignin is racemic. As a result, lignin is formed by two "enantiomeric" groups of strings [154]. Unfortunately these are only calculations, not backed up by evidence yet and the discussion can go on.

Conclusion

There is not much left to be said here, the two theories and the most compelling arguments for both sides has already been discussed and everyone should draw his own conclusions. For a more detailed discussion we suggest a couple of reviews written from both point of views [65,146,155,156]. Unless where otherwise specified the presented evidence was taken from those reviews. We wish to remember once again that disregarding every personal opinion on the topic, the matter is still debated. It is better to wait for more convincing evidence before dismissing one of the two options.

1.3.4 APPLICATIONS

After this digression, not so pertinent to lignin biosynthesis, the main topic can be further discussed. It is time to consider the potential uses of the knowledge gathered by perturbing the monolognols biosynthesis pathway, i.e. transgenic plants. This is a very young field and what is under discussion nowadays, are only potential applications or promising options. Before transgenic plants can be employed in industry, a great deal of research has to be performed. It is important to understand what possible repercussion on the environment transgenic plants could have and how they would affect biological equilibrium. Even more studies need to be done if transgenic plants will be employed as food for cattle. All currently envisaged applications are aimed to a reduction in lignin content or to an easier lignin removal. Even with the knowledge acquired from monolignol and lignin biosynthesis, it is still hard to foresee a possible way to convert lignin into something useful, but only to reduce the harm it does to other applications.

F5H upregulation, key to an easier pulping.

Between all of those described above, upregulation of F5H is the genetic modification that has arosen most interest. Many research groups focused their attention on this particular enzyme because of the promising variations in lignin structure. Upregulation of F5H produces an increased amount of syringyl units (**33**) in lignin, due to the most abundant production of its precursor, 5-hydroxyconiferyl alcohol (**63**). The reason for the interest is that S units are much more likely to couple through *β*-O-4 (**34**) bond; due to the unavailability of position 5, *β*-5 (**36**) bonds and dibenzodioxocine rings (**38**) cannot be formed. This produces a linear lignin, much more degradable because of the abundance of *β*-O-4 bonds, easily broken during pulping. Therefore an increased S amount in lignin will allow a more efficient pulping with softer and cheaper reaction conditions. As reported before, when COMT activity is not high enough, incorporation of 5-hydroxyconiferyl alcohol in lignin is observed, with appearance of the benzodioxane bond (**70**). This is the case for example of *Arabidopsis* [157]. Benzodioxane is hard to cleave and considered undesirable for wood pulping. Therefore a better plant with higher COMT activity was searched. The choice fell on poplar, a fast growing tree that displayed over 97% of S units in F5H upregulated lines. Several tests have been performed of this transgenic plant. Pulping tests gave really positive outcome, with higher yields and better efficiency [158], structural studies revealed lignin chain shorter than normal with a lower β-O-4 content than expected [159]. Culture tests in greenhouses are being conducted to verify that this transgenic poplar is biocompatible and that it can actually grow properly without structural malfunctions. General resistance to outdoor conditions and growth rate are monitored [160]. Additional data need to be gathered, but the potential for industrial use of this plant is really interesting.

Other applications

Forages growth is another activity that could have beneficial effects from genetically modified lignin. Lignin-polysaccharide cross links, most likely the ones formed by ferulic acid (**49**), are responsible for low digestibility. Therefore a lignin with lower ferulate content would increase intake potential and energy availability of forage crops, resulting in better food for cattle. With current knowledge, the only way to reduce ferulate cross links is to downregulate lignin production, which usually leads to small and malformed plants [161]. Therefore additional studies are necessary to understand how to regulate cross-links

in lignin. In any case before using transgenic plants as cattle food deep and accurate studies on metabolism are necessary, together with eco-compatibility ones.

Genetic regulation of enzymes has been performed also on softwood conifers. The overall results are partially consistent with hardwood tests and partially unexpected. Enzyme regulation has been performed on pine for 4CL, CCOMT and CCR [162-164]. Reduction of lignin content and changes in lignin composition were registered as expected, but other phenotypic effects were observed as well. Modified carbohydrate metabolism and changes in wood/bark ratio are some of the most unexpected effects measured in transgenic softwoods. This results point out that gymnosperm physiological complexity is greater than expected, most likely lignin plays a more relevant role in conifers development than in angiosperms. This role will need to be further investigated before it will be possible to effectively modify softwood [162].

1.4 Lignin model compounds

At the end of chapter 1.2 (p.43 section 1.2.5) two main approaches to lignin knowledge were reported: biosynthesis study, which has just been covered, and model compounds. The latter will be discussed here. A model compound is a simple molecule, synthesized or otherwise obtained, which reacts in a similar way to the more complex compound of interest. Basically, it is a simplified version of the molecule that needs to be studied. With such a molecule, for example, it is possible to perform easier and more precise analysis, or to test the specific reactivity of a small portion of a bigger molecule. It goes by itself that, with a polymer such as lignin, the simplification provided by model compounds is more than needed. Model compounds in lignin are mostly synthetic monlignols dimers or oligomers, with two main applications. In early work, model compounds were used to confirm and back up analytical data obtained from natural lignin. Degradative studies were performed on both models and lignin samples to confirm the presence and identity of specific bonds, structures and constituents. Later on the same strategy has been applied to more advanced analysis methods, such as NMR. Model compounds are employed to obtain clearer spectra, without interferences or overlapping peaks, in order to properly assign each signal and identify specific peaks for each structure [165,166]. Our knowledge of lignin has been widely improved by the analysis of model compounds, and peak assignment of in vivo lignin made much easier. The other main field of application for model compounds is

the reactivity test for each lignin linkage. Most applicative studies conducted on lignin are aimed at its decomposition, all kind of reactions have been attempted and studied, with the purpose of better converting lignin to smaller constituents [167]. By using the appropriate model compound it is possible to study each linkage individually, which allows more specific tests and much more understandable results. Model compounds are very useful, because they can simplify problems otherwise too complicated to be solved. The most important feature of a model compound is its similarity to the original molecule; it is pointless to perform very accurate analysis on a molecule which is not related to the real target of the analysis. Sometimes it is hard to determine whether a molecule and its model are similar enough. In any case, even with the most refined model, it is important to keep in mind that it is just a model, and that it might not respond exactly as the original molecule.

DHP

The most important features of dehydrogenation polymer have already been described (p. 52 section 1.3.3); here it will be discussed with more technical details. Leaving aside every dispute about DHP similarity to natural lignin, only the data will be presented, and it is left to the reader to decide whether DHP is a suitable model for lignin or not. Anyway, since in the past DHP has been assumed to be identical to lignin, it is now important to make it clear that even if they are similar, they are not the same thing. DHP is obtained after radical oxidation of monolignols, just like lignin, what differs are the other reaction conditions. The dehydrogenation theory was presented by Erdtman in 1933 [168], when he found out that reaction of monolignols with ferric chloride ($FeCl_3$), or mushroom oxidases, gave phenylcoumaran (**36**) (β-5) as product. Since phenylcoumaran structures had earlier been found in lignin, Erdtman proposed that lignin was also formed by dehydrogenation. More convincing evidence to back up the dehydrogenation theory was found and reported over the years. Some years later, further degradation studies were performed on coniferyl alcohol (**29**) reacted with $FeCl_3$. Treatment with hot alkali followed by methylation and permanganate oxidation produced the same degraded molecules as lignin [169]. Finally, reaction of coniferyl alcohol with enzymes such as oxygenases, laccases and peroxidases produced a polymer which showed some similarities with Björkman lignin [170,171]. This polymer was called dehydrogenation polymer, DHP. In early work, DHP was prepared with what is called the Zulauf method; all the reagents are put together in a batch and then reacted. The outcome is a DHP with very few similarities with lignin, the most abundant

linkage is β-5, β-O-4 (**34**) concentration is very low and some bonds are not present at all. The reason is that monomers concentration favors monomer-monomer coupling, while monomer-polymer addition is thought to be the main phenomenon in lignin constitution. Therefore DHP is nowadays synthesized with the Zutprof method, which consists in slow addition of monomers into a solution containing enzymes and oxidants. With this method β-O-4 concentration rises, but the linkages ratios are still different from the ones in lignin [172]. In recent years more sophisticated synthesis procedures have been proposed. In order to achieve the high dilution assumed for in vivo process, coniferyl alcohol has been diffused in the reaction mixture through a membrane. With this method monomer dimerization has been drastically reduced and similarity to lignin improved. Once again, even with this method, DHP and natural lignin are very similar but not identical [150].

Apparently the choice of oxidant does not strongly affect the outcome of the reaction. DHP has been obtained with inorganic oxidants, such as $FeCl_3$ or MnO_2 and different enzymes, such as Oxidases and laccases with oxygen, or peroxidases with H_2O_2. Currently Horseradish peroxidase (HRP) is perhaps the most employed enzyme. Another factor that is currently under investigation is the influence of carbohydrate presence on DHP (and lignin) growth, as has been discussed in (p. 31 section 1.2.2). Also monolignols oxidation potentials have been researched [173]. Each monolignol has a different oxidation potential, which varies with pH and with the linkages formed. It is believed that the oxidation potentials play a role in defining the structure of lignin, that it somehow helps regulating the kind of bonds formed. No successful attempts to apply this knowledge to DHP synthesis have been made, but it is possible that the control of this feature could allow another improvement in DHP's similarity to lignin.

Among other things, DHP helped scientists to understand how cell wall works [174], and to study linkages between lignin and cellulose (**4**), along with the opportunity to break them and degrade lignin [175,176]. Surprisingly in vitro DHP also shows some sort of long range ordering, apparently macro aggregates of DHP are formed even without sugars or other entities [177].

Released suspension cell culture lignin (RSCL)

RSCL is a new model compound for lignin, more evolved than DHP. When lignifying cells are grown in a suspension culture, they release lignin in the medium. This lignin can be gathered, purified and analyzed. RSCL offers the unique possibility of performing analysis on only slightly modified lignin. Extraction and purification processes are much less invasive compared to the ones necessary to purify natural lignin. In fact, RSCL lignin is already free from cellulose (**4**), hemicellulose (**5**), and most of the other cell wall components. Many different cultures, with several media and conditions have been grown and analyzed [178]. The overall result is that RSCL is substantially different from extracted in vivo lignin, apparently not similar enough to be a suitable model [179]. It is probably due to the fact that the conditions of polymer growth, outside the cell wall matrix, are extremely different from the conditions inside. In particular RSCL display an excessive amount of β-β (**40**) linkages, with a too low amount of β-1 (**41**). If further modification of the reaction conditions will allow us to produce RSCL similar enough to natural lignin, this would probably produce a meaningful advancement in our knowledge of lignin. With such an easily purifiable lignin it would be possible to perform much more accurate analysis.

1.4.1 RESEARCH TOPIC

Founding hypothesis

Our research is based on DHP study, and aims at shading some light on lignin formation mechanism. If the combinatorial theory for lignin biosynthesis is right, then our hypothesis is that there are some closed loops between different lignin branches. Some linkages in lignin (4-O-5 (**39**), dibenzodioxocin (**38**)) are responsible for branching, i.e. the growing of two distinct lignin chains from a single one. We believe that, since intramolecular reactions are faster than intermolecular ones, those two branches will eventually react together and form a closed loop, as shown in Figure 1.17. Many reaction of this kind are possible, from radical coupling in every available position to nucleophilic attack on a quinone methide (**35**) by any free alcohol. These reactions would end the growth of the lignin polymer for both chains. On the other hand, if lignin growth is controlled by enzymes, it would be harder for both growing branches to be accommodated in the enzyme active site and therefore those loops would not be present. Our project is to polymerize DHP and look for evidence for the existence of the loops.

Once found it would be necessary to identify specific peaks, markers or any clear sign of their existence, in order to perform the same analysis on natural lignin and discover whether the loops are present or not.

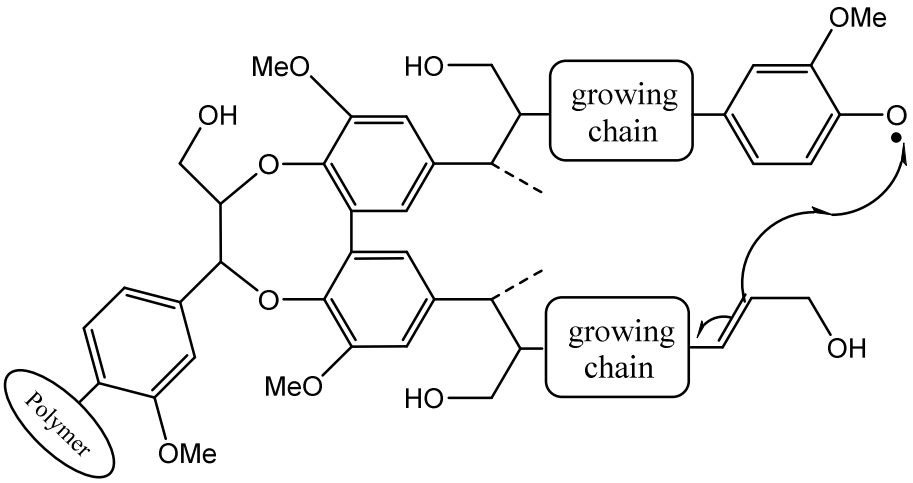


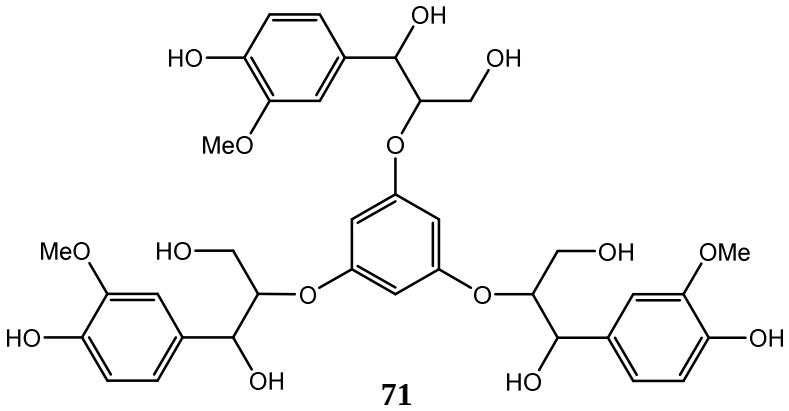
Figure 1.17shows an example of the hypothesized closed loop. The dibenzodioxocin ring acts as a branching point in lignin. Two of the growing chain, both oxidized, interact together to form a β-O-4 bonds and close the ring.

Our project

Detection of the loops will be very hard to achieve, therefore it is necessary to increase the chances for this ring closing reaction to happen. By raising the concentration of closed loops in the sample, it will be easier to detect them. The concentration of 4-O-5 (**39**) and dibenzodioxocin (**38**) linkages is too low in both lignin and DHP to offer us a good chance to achieve our goal, therefore a new approach to the problem had to be found. When a branching point in lignin is formed, three distinct growing chains are present: The original one, growing before the branching, and the two chains formed there (Figure 1.17). By increasing the number of branching points we expect to obtain a higher number of loops. The way to obtain more branching points was suggested by the polymer chemistry field, in particular by star polymers. Star polymers are getting more and more attention lately, and they are achieving good applicative results in several fields [180]. Star polymers are just normal polymers that use a multi branched molecule as a starting polymerization center. Therefore the polymer instead of growing in only one sense spreads in as many directions as the number of the central branches. Star polymers can be synthesized in different ways, just like normal polymers, and every sort of copolymer can be prepared [181]. Star polymers are being tested for applications in medical field, for slow- release drugs, as ion

traps, as flocculants for easier separation in industrial processes and in many other applications [182-185].

With the same idea, we decided to synthesize a new, three branched, model compound for lignin, and to use it as a polymerization center for DHP growth. Without a branched polymerization center, lignin would mostly grow linearly, in the two opposed direction senses. This would hardly lead to an intramolecular reaction. On the other hand three growing branches, separated by a ≈120° angle, will more likely interact with each other. The model compound we decided to synthesize and use for the following polymerization is shown in Figure 1.18. To our knowledge, this molecule is new and it has never been synthesized before. This molecule will be called model compound 3 (**71**) (MC3).



71

Model compound 3

Figure 1.18 shows our target molecule, MC3. Our model is composed by three coniferyl alcohols linked in a β-O-4 fashion to a central aromatic ring, the start of three lignin growing chains. A three branched, star, lignin model compound.

Related research

It is possible that this new kind of model compounds will open new chances in lignin research, for example in the study of the 3D spatial growth of the polymer. The interactions between growing polymer chains could be studied in order to better understand the relation and influence of lignin with itself. The increased amounts of branching point will give a better understanding of how lignin actually develops in space, as opposed to the almost linear filament that could be obtained with normal DHP synthesis. The study of 3D lignin interaction might be helpful to prove the template polymerization hypothesis (p. 56). Another interesting use for this new three branched polymer could be the growth of DHP in thin layers. Attempts have already been made with classical model compounds, with not

excellent results [186]. In fact DHP hardly grows in layers, the surface is not entirely covered and a monolayer is not obtained. It is possible that our new model compounds will provide a better surface coverage, which could allow the synthesis of a proper monolayer. This would be a very interesting achievement for lignin chemistry. For starters it would be possible to perform surface analysis on DHP, which would increase our knowledge of linkages distribution and formation. In addition to that, a whole field of applications for coniferyl alcohol (**29**) would be available. Modified layers of DHP could be very interesting materials, derivatization of coniferyl alcohols and other monolignols might allow the tailoring of the monolayer properties according to our needs. Of course, this is just a prospect for future research, as it is unknown if it is even possible to grow DHP in layers.

Project outline

Before moving on to the description of our work, it is appropriate to summarize what said so far about our project and to draw a simple scheme of the purpose of the research.

1. Synthesis of new, three branched, lignin model compounds. The idea came from star polymers. As this kind of molecules have never been synthesized the whole process need to be tested and optimized.
2. Polymerization tests. It is necessary to prove that the synthesized model compounds will act as polymerization centers.
3. Analysis on DHP, to possibly detect closed loop structures. Identification and characterization.
4. Eventual further studies (3D growth or monolayer synthesis)

2 Experimental

This chapter is divided into three main parts. The first part is dedicated to the description of the analytical tools employed. The following parts report the procedures employed during the synthesis of the model compounds and the following polymerization, respectively. Only the best procedure achieved for each step is reported here, leaving aside all the attempts that were necessary to find it.

2.1 Analysis

Several analysis tools were employed during this project. The instrument employed and the most important experimental procedures are reported here.

2.1.1 NUCLEAR MAGNETIC RESONANCE (NMR)

NMR spectra were recorded on Varian Inova 500, Varian Mercury Plus 300 and Varian Unity-300 spectrometers (1H: 500 and 300 MHz, and 13C: 125 and 75 MHz, respectively) using $CDCl_3$, D_2O, DMSO-d6 or acetone-d6 as solvents. The 300 MHz instruments were normally employed for routine analysis, while the 500 MHz machine was used only on relevant samples to obtain a better resolution. Two-dimensional NMR techniques (HSQC, HSQC-TOCSY and HMBC) were used for the identification of products using standard pulse sequences provided by the manufacturer for acquisition and data processing.

NMR samples preparation

Typically NMR samples were prepared by dissolving 20-30 mg of the sample in the chosen deuterated solvent. The 2D and ^{13}C experiments widely employed during the project require a high amount of sample to be recorded in a reasonable time. Solubility was a big issue for both analysis and synthesis; several deuterated solvents were employed for NMR. The most employed were $CDCl_3$, DMSO D_6 and acetone D_6. Sometimes, because of the low solubility or low yield, small samples (<5mg) had to be analyzed.

2.1.2 SIZE EXCLUSION CHROMATOGRAPHY (SEC)

The molecular weight distribution analysis was performed using Waters 990 equipment including degasser, auto sampler and column oven (Waters 717 plus Auto sampler, Waters 515 HPLC pump, Biotech Model 2003 Degasser) with 50 μL injection. Separation was done using Waters Styragel HR1, HR2 and HR4 columns in 30 °C eluting with THF 0.8 mL/min with 1% toluene as internal standard. Waters 2487 Dual Absorbance Detector, at UV wavelength of 254 nm and refractive index (RI) detector were used for detection. The molecular mass was calibrated using polystyrene standards. Millennium 32 GPC software (Waters) was used for data processing to obtain as numerical output M_n (number-average molecular weight), and M_w (weight-average molecular weight). The polydispersity PDI (M_w/M_n) has been calculated as well.

SEC samples preparation

For the preparation of SEC samples1-2 mg of product were dissolved in 1 mL dry THF, with 1% of toluene as a reference. The solution was then filtered in the SEC vial using a 0.45 μm Acrodisc GHP Membrane HPLC filter.

2.1.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

HPLC was performed using an Agilent 1200 series HPLC machine on a C18 stationary-phase column Zorbax Eclipse RP XDB-C18 (2,1×100 mm, 3,1 μm) at 40°C. The mobile phase usually was water : methanol (50:50). Detection was via UV 7 spectrometry, chromatograms were produced from 270 nm monitoring, with additional information provided at 254 nm and 280 nm.

HPLC samples preparation

To prepare an HPLC sample, 1-2 mg of the desired product were dissolved in 1 mL of dioxane or methanol, according to solubility. The solution was then diluted 1:10 with a mixture MeOH/H_2O, in order to obtain a final solution 1:1 organics/water. Then, 1 mL of the sample was filtered with a 0.45 μm Acrodisc GHP Membrane HPLC filter and put in a HPLC vial.

2.1.4 THIN LAYER CHROMATOGRAPHY (TLC)

TLC was performed on Merck silica gel 60 F254 plates, in various solvent systems. Due to the high aromaticity of the reactants, the spots were visualized with ultraviolet light only. The solvent system varied depending on the separation desired and on the molecules solubility. TLC has been employed to test the best eluent for each chromatographic column, and to follow each reaction. Reaction times for routine operations were decided according to TLC results.

2.1.5 BIOTAGE SEPARATION

When the solvents employed allowed it, Biotage separation was used instead of column chromatography. It was performed on a BiotageIsoleraSpektra 4 using a 10g SNAP Ultra cartridge. The collection wavelength was 280 nm and the monitoring wavelength 254 nm. Because of the employed wavelengths, this instrument could only be used when toluene was not present in the eluent mixture. The parameters (solvent gradients, eluent amount, flow rate, etc.) were suggested by the instrument on the basis of the TLC results. Biotage separation was mostly used during MC3 synthesis, for purification after the first reaction step. The products fractions were collected automatically according to the UV peaks detected.

2.2 Synthesis

The synthesis of three model compounds for lignin, with increasing complexity and similarity to lignin structures, was attempted in this project. Each one will be discussed individually. Detailed discussion and analysis of each compound will be given in the results and discussion chapter. Even if these compounds have never been synthesized before, the reactions employed are commonly performed in organic chemistry and in lignin model compounds synthesis [60,187-191]. The reaction steps needed to be tested for results and optimized. The synthesis pathway was based on former work in lignin model compounds chemistry and on our laboratory experience.

2.2.1 MODEL COMPOUND 1 (MC1)

The first synthesized model compound and the reaction scheme are shown in Figure 2.1 1For both MC1 (**75**) and MC2 (**78**) synthesis, the necessary amount of 1-(4-(benzyloxy)-3-methoxyphenyl)-2-bromoethan-1-one (**73**) (BBAVone) had already been synthesized in our laboratory.

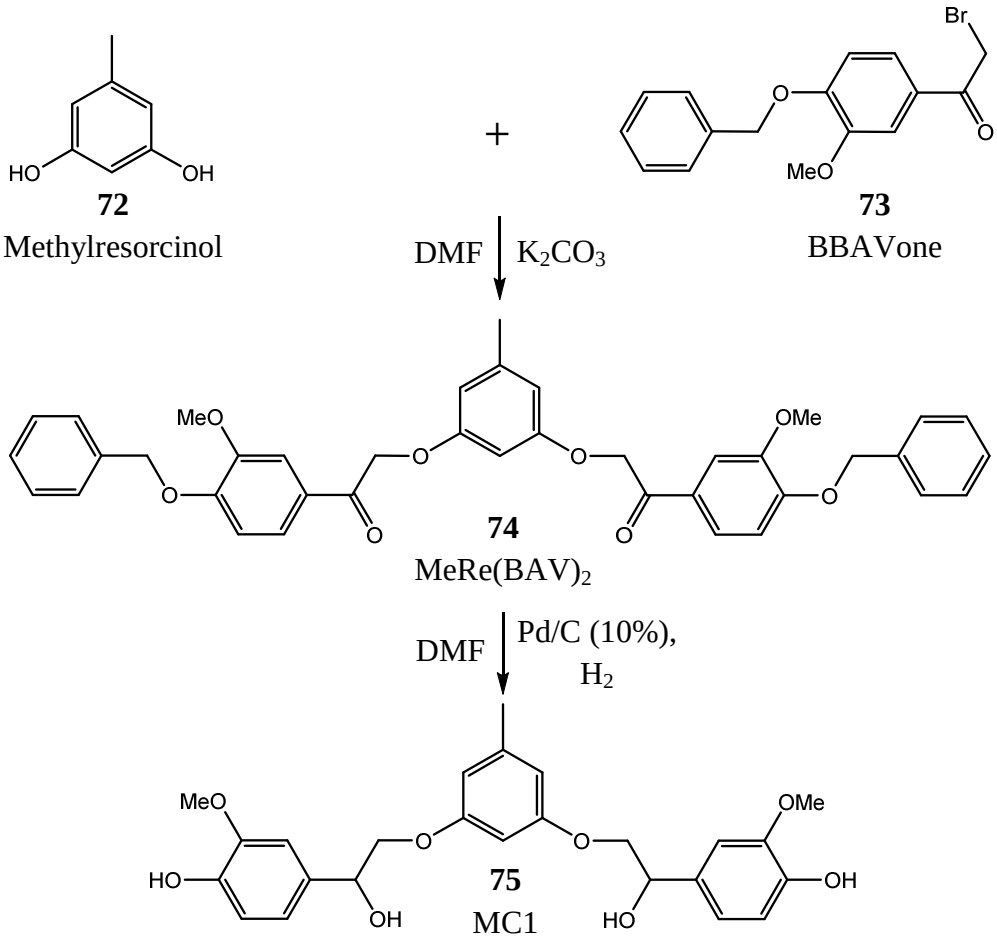


*Figure 2.1Shows the reaction scheme to obtain the target molecule MC1 (**75**).*

Step 1: nucleophilic addition

Typically, for the first step of the reaction, 15 mg of 5-methylresorcinol (**72**) (MeRe, 0.106 mmol, Mw=142.15 g/mol) and 70.7 mg of 1-(4-(benzyloxy)-3-methoxyphenyl)-2-bromoethan-1-one (**73**) (BBAVone, 0.211 mmol, Mw=335 g/mol), in stoichiometric amounts, were dissolved in 5 mL of DMF, together with 43.8 mg of K_2CO_3 (0.316 mmol, Mw=138.21 g/mol). The amount of K_2CO_3 is in a 150% in excess, three times the number of MeRe moles. The excess was considered necessary because of the low strength of the base. The reaction mixture was left under stirring and reflux overnight, at room temperature (RT). The reaction was stopped by pouring 10 mL of H_2O and 30 mL of ethyl acetate (EtOAc) in the mixture. The two phases were stirred together for 10 min and the organics extracted. The two phases were separated and the organic phase was then washed with 2M NaOH and brine, the EtOAc solution dried on Na_2SO_4 and the solvent evaporated. The crude solid was recrystallized for purification. The product was dissolved in hot toluene (70°C) with the final addition of some drops of pentane, until the first traces of precipitation were observed. The solution was left overnight in the fridge, the crystallized product filtered and washed with cold solvent. The obtained pure product, 2,2'-((5-methyl-1,3-phenylene)bis(oxy))bis(1-(4-(benzyloxy)-3-methoxyphenyl)ethan-1-one) (**74**) (MeRe$(BAV)_2$) (Mw=632 g/mol, yield 36%), was then hydrogenated to complete the synthesis.

Step 2: reduction and deprotection

For the hydrogenation step, 50 mg of MeRe$(BAV)_2$ (**74**) (0.079 mmol) were dissolved in 25 mL of DMF. The catalyst chosen for the hydrogenation was Palladium on activated charcoal, 10% Pd basis (based on dry substance) moistened with water (~50% water as stabilizer) (Aldrich). The catalyst was added in the same amount as the reactant, 50 mg. The reaction mixture was left under vigorous stirring and H_2 overnight, then filtered and the solvent evaporated. It was typically hard to get rid of all the DMF employed as solvent, therefore after evaporation the products were dissolved in EtOAc. The solution was then washed with water, brine, dried on Na_2SO_4 and the solvent evaporated. MC1 (**75**) (Mw=456 g/mol, yield 95%) was obtained in solid state. Each product obtained in the solid state was typically left to dry under vacuum, before proceeding to further steps or analysis.

2.2.2 MODEL COMPOUND 2 (MC2)

The second model compound is shown in Figure 2.2 along with the reaction scheme.

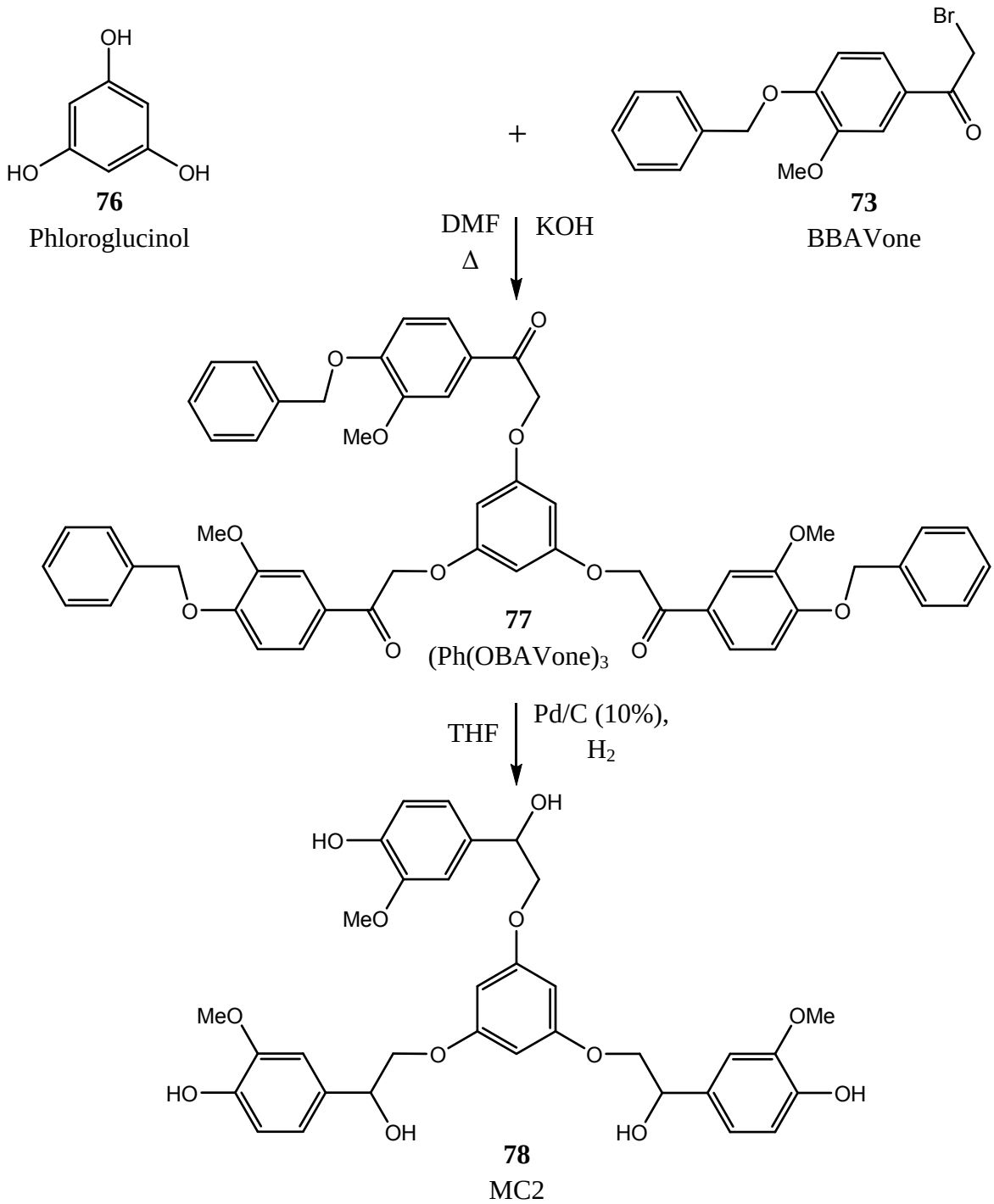


*Figure 2.2 Shows the reaction scheme to obtain the target molecule MC2 (**78**).*

Step 1: nucleophilic addition

For the first step of this synthesis 61.4 mg of phloroglucinol (**76**) ($Ph(OH)_3$, 0.487 mmol, Mw= 126.11 g/mol) and 83.7 mg of ground KOH (0.149 mmol, Mw=56.11), in stoichiometric amount, were dissolved in 1.5 mL of DMF. They were left to react under reflux at 40°C for 30 min. Then 500 mg of BBAVone (**73**) (0.149 mmol, Mw=335 g/mol), dissolved in 2 mL of DMF, were slowly added over 30 min. The reaction was left under reflux and stirring at 40°C overnight. The reaction produced a solid and a liquid phase. After filtration the liquid phase was discarded (more details at p. 71 section.2.2.2), and the solid phase washed with H_2O, 2M HCl, and EtOAc. This phase was insoluble in most solvents. The solid phase (yield 21.3%), containing 2,2',2''-(benzene-1,3,5-triyltris(oxy))tris(1-(4-(benzyloxy)-3-methoxyphenyl)ethan-1-one) (**77**), ($Ph(OBAVone)_3$, Mw= 888 g/mol) was dried in the vacuum pump before moving to the hydrogenation step.

Step 2: reduction and deprotection

For the hydrogenation step, 100 mg of $Ph(OBAVone)_3$ (**77**) (0.113 mmol, Mw= 888 g/mol) were partially dissolved in 100 mL of THF. To the suspension were added 200mg of Pd/C (10%) catalyst. The reaction was left overnight under H_2 flow and vigorous stirring. After the reaction, almost all the starting $Ph(OBAVone)_3$ was hydrogenated and the alcohols deprotected. The product was soluble in THF; it was therefore safe to filter out the mixture to eliminate the catalyst. After filtration THF was evaporated, and a solid mixture obtained. The solid was washed with toluene and $CHCl_3$ to remove impurities, and then dissolved in EtOAc. The solution was washed with water and brine, dried over Na_2SO_4 and the solvent evaporated. Pure MC2 (**78**) (Mw= 624 g/mol, yield 33.5%) was obtained at the end of the procedure.

2.2.3 MODEL COMPOUND 3 (MC3)

The third synthesized model compound and its reaction scheme are shown in Figure 2.3.

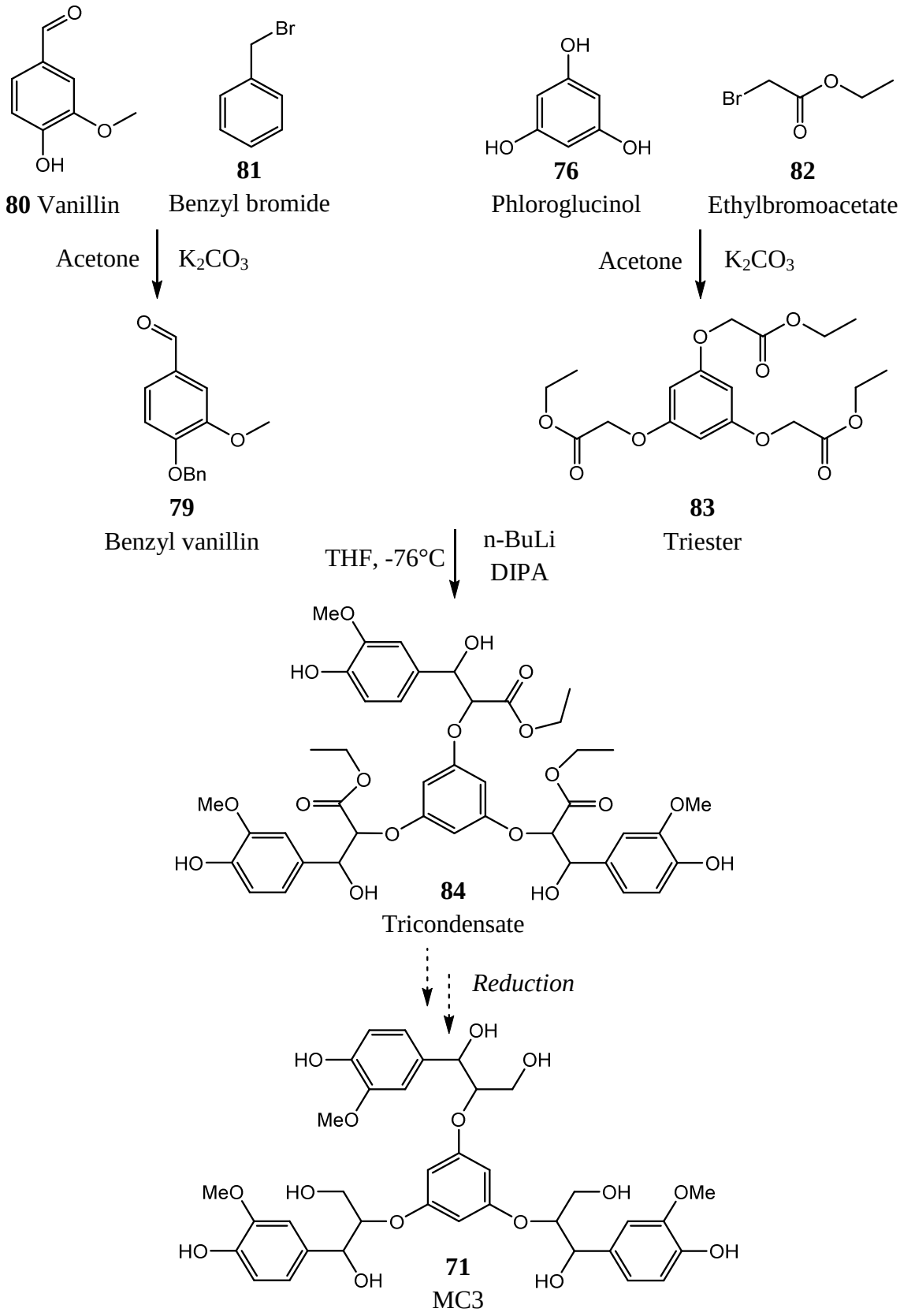


*Figure 2.3 Shows the reaction scheme to obtain the target molecule MC3(**71**).*

Step 1: Benzyl vanillin synthesis

The synthesis procedure of benzyl vanillin (**79**) (Mw= 242 g/mol) was already established and therefore has only been performed once. 26 g of vanillin (**80**) (0.171 mol, Mw= 152.15 g/mol) were dissolved in 125 mL of EtOH and 20 mL of benzyl bromide (**81**) (0.168 mol, Mw= 171.03 g/mol, ρ= 1.438 g/cm^3). 24 g of K_2CO_3 (0.174 mol, Mw=138.21 g/mol) were added to the mixture. The reaction was left under reflux for 3 h. The warm mixture was poured into 1 L of NaOH 0.5 M, the solids were filtered and recrystallized from EtOH.

Step 2: nucleophilic addition

Typically, for the synthesis of MC3 (**71**), 2 g of phloroglucinol (**76**) (0.016 mol, Mw= 126.11 g/mol) and 8g of ethyl bromoacetate (**82**) (0.069 mol, Mw= 176 g/mol, ρ= 1.51 g/cm^3) were reacted in 50 mL of acetone with 6.6 g of K_2CO_3 (0.047 mol, Mw=138.21 g/mol). The reaction was left under reflux overnight at 90°C. After cooling, the solid was filtered and washed with acetone to extract the product from the solid waste. The combined organic solution was evaporated, and the obtained organic solid dissolved in 50 mLof EtOAc. The organic phase was washed with 2M NaOH and brine, dried over Na_2SO_4 and the solvent evaporated. The residual benzyl vanillin present in the solid was eliminated by silica flash column chromatography, with a 5:2 pentane/EtOAc mixture as eluent. Because of the low solubility the solid was first dissolved in a 1:1 pentane/EtOAc mixture, then dried and adsorbed on silica. The impregnated silica was then added at the top of the chromatographic column. The pure product of interest, triethyl 2,2',2"-(benzene-1,3,5-triyltris(oxy))triacetate (**83**), (triester, Mw= 384 g/mol yield 46.8%) was collected and the solvent evaporated.

Step 3: aldol condensation

The following step consisted in the condensation of the obtained triester (**83**) with the previously synthesized benzyl vanillin (**79**). 1.68 mL of diisopropylamine (DIPA, 12 mmol, Mw= 101.19 g/mol, ρ= 0.722 g/cm^3) were diluted in 5 mL of dry THF under argon and cooled to 0°C in an ice/salted water bath. Then 6.25 mL of n-butyl lithium in hexane (n-BuLi, 10 mmol, 1.6 mol/L) were slowly added to the reaction mixture over 15 min. The mixture was left to react for 30 min, always at the same temperature and under inert atmosphere. The reaction mixture was then cooled in an ethanol-dry ice cooling bath. When the reaction reached -70°C, 1 g of triester (2.6 mmol, Mw= 384 g/mol) dissolved in 5 mL of dry THF, was slowly added over 40 min. the temperature was kept stable during

the addition. Without delay, 1.94 g of benzyl vanillin (8 mmol, 242 g/mol) in 15 mL of dry THF were added to the reaction over a period of 1h. After the end of the addition the reaction was left to stir for ≈2h, always under argon. The mixture was then quenched in a saturated NH_4Cl solution and neutralized with 2M HCl. 30 mL of EtOAc were added to the reaction mixture to extract the organics. The EtOAc solution was then separated and washed with 2M HCl, a saturated solution of $NaHCO_3$ and brine, dried over Na_2SO_4 and the solvent evaporated. The obtained solid contained a high amount of impurities. Purification has been attempted and partially achieved with a silica flash chromatographic column, using a mixture 2:1 toluene/EtOAc as eluent. The purification of the product, triethyl-2,2',2"(benzene-1,3,5-triyltris(oxy))tris(3-hydroxy-3-(4-hydroxy-3-methoxyphenyl)propanoate) (**84**), (tricondensate, Mw=1110 g/mol) was only partial, and the yield extremely low (1-4%), therefore the synthesis of MC3 (**71**) has been abandoned.

2.2 Polymerization

DHP polymerization has been conducted under a variety of conditions. All variables are reported in table 2.1. The general procedure for each step is described below.

Buffer preparation

A buffer at pH 6 of citric acid and Na_2HPO_4 was required for the polymerization. A solution of citric acid, 100 mL 0.1M (Mw= 210.14 g/mol) and 100 mL of Na_2HPO_4 solution 0.2M (Mw=177.99 g/mol) were prepared. 63.15 mL of Na_2HPO_4 solution and 36.85 mL of citric acid solution were mixed together. The pH was measured with a pH meter and adjusted to 6 by slow addition of the same solutions. The same procedure, with 61.45 mL of citric acid and 38.53 mL of Na_2HPO_4 as starting amounts, was performed to obtain a pH 4 buffer.

Model compounds and coniferyl alcohol

Three model compounds were used as starting polymerization centers: MC1 (**75**), 5-methylresorcinol (**72**) and phloroglucinol (**76**). Even if MC2 (**78**) was synthesized and purified, the yield of the reaction was too low to obtain a proper amount of the molecule, especially if a series of tests had to be performed. The coniferyl alcohol (**29**) (Mw= 180.2 g/mol) employed had already been synthesized in our laboratory.

Model Compound	M.C. mmol	M.C. mg	CA mmol	CA mg	H_2O_2 mL	H_2O_2 mmol	pH	Addition time	Liquid added (mL)
MeRe	0.703	100	-	-	2	2.2	6	1h	2
MeRe	0.703	100	0.703	126.8	1.5	1.65	6	1h	2
MeRe	0.703	100	1.406	253.6	2.5	2.75	6	1h	5
MeRe	0.703	100	2.812	507.2	4.8	5.28	6	1h	5
MeRe	0.703	100	0.703	253.6	2.8	3.08	4	1h	5
$Ph(OH)_3$	0.793	100	-	-	1.6	1.76	6	1h	2
$Ph(OH)_3$	0.793	100	0.793	143.02	2	2.2	6	1h	2
$Ph(OH)_3$	0.793	100	2.381	429.06	4.9	5.39	6	1h	5
$Ph(OH)_3$	0.396	50	2.378	428.07	4	4.4	6	1h	5
MC1	0.110	50	0.219	39.52	0.3	0.33	6	-	2
MC1	0.132	60	-	-	0.18	0.198	6	1h	2
MC1	0.132	60	0.132	23.7	0.36	0.396	6	1h	2
MC1	0.132	60	0.264	47.4	0.5	0.55	6	1h	2
MC1	0.132	60	0.528	94.8	0.9	0.99	6	1h	2
Test 0	-	-	1.110	200	1.5	1.65	6	1h	2

Table 2.1 resumes the experimental parameters adopted for each polymerization experiment.

Polymerization procedure

The desired amount of model compound, reported in table 2.1, was dissolved in 3 mL of acetone. 2-3 mg of HRP-enzyme (Serva, 897 U/mg) were dissolved in 1 mL of distilled water. The two solutions were mixed together with 6 mL of buffer. In order to prevent denaturation of the enzyme, the acetone and the HRP solution were never directly mixed together. The indicated amount of coniferyl alcohol (**29**) was dissolved in 2-5 mL of acetone and the corresponding quantity of H_2O_2 (1.1 M) was diluted in the same amount of water. The H_2O_2 was added with ≈150% stoichiometric excess over the free phenols, from both the model compound and the coniferyl alcohol. Both coniferyl alcohol and H_2O_2 were slowly added to the reaction mixture in 1h time, with the help of an automated syringe pump (except for one attempt). The reaction was left under weak stirring overnight. The reaction usually produced a solid and a liquid phase. To stop the reaction 20

mL of EtOAc were poured in, and the low molecular weight fraction of DHP was extracted. The mixture was filtered and the solid heavy fraction collected, washed with water and dried under vacuum. The organic phase, where the light fraction was dissolved, was washed with water and brine, dried over Na_2SO_4 and the solvent evaporated. After drying, both fractions were acetylated individually. Typically the whole amount of DHP gathered was acetylated, with few exceptions. The employed acetylation reaction conditions were harsher than necessary to assure complete acetylation in every case. The obtained DHP was dissolved in 2 mL of pyridine and 2 mL of acetic anhydride, and left under reflux overnight at 50°C. The reaction was quenched in EtOH and the solvent evaporated, for three times. In order to completely remove pyridine as azeotrope the same procedure was conducted using toluene as cosolvent and finally with $CHCl_3$. The product was then dried under vacuum.

3 Results and Discussion

In the first part of the chapter, the synthesis of each model compound is discussed individually, and the relevant data presented and discussed. The second part of the chapter is devoted to the analysis of the polymerization. The most relevant NMR spectra are included in the text; peak assignments have been performed with the help of 2D NMR experiments.

3.1 Synthesis

3.1.1 MC1

The reaction scheme for MC1 (**75**) synthesis is presented in Figure 2.1 (p. 68). This synthesis was the easiest and the one where the best results were achieved. As this compound has only two branches, a possible objection is that it will promote a linear lignin chain, with the only branching point provided by the rare 5-5 (**37**) and 4-O-5 (**39**) linkages. We believe that since the angle between those growing chains is, supposedly, 120° and not 180° the interactions between different lignin segments will happen nonetheless.

Step 1: nucleophilic substitution

The most successful synthetic procedure to obtain MC1 (**75**) was described earlier (p. 68 section 2.2.1). The most relevant attempts to optimize the first step of the reaction are listed in table 3.1. Reaction at room temperature achieved a crude yield of 90.4%. In previous attempts, with same conditions and temperature of 40°C the crude yield was ≈ 80 %. Worst results were obtained using KOH and a phase transfer catalyst (t-$BuNHSO_4$), always at 40°C, with yield of 61.0%.

Attempt n°	Base employed	Base amount	Temperature (°C)	Other reactant	Crude yield (%)
1	K_2CO_3	stoichiometric excess (150%)	40	none	81.7%
2	KOH	stoichiometric excess (150%)	25	t-$BuNHSO_4$	61.0%
3	K_2CO_3	stoichiometric excess (150%)	25	none	90.4%

Table 3.1 resumes the reaction conditions tested for the nucleophilic substitution step for molecule MC1, and the relative crude yields.

A problem for this reaction was the purification of the crude product. Attempts have been made with column chromatography, using a 6:1 toluene/EtOAC mixture as eluent, with purification yields of 20%. More significant results were obtained using the low solubility of the compound for recrystallization. Attempts with EtOH and toluene as solvents achieved purification with 40% yield. The toluene temperature during the dissolution was kept lower than the boiling point for fear of thermal degradation, but no tests at a higher temperature were performed. The NMR spectrum of the crude product suggests that the desired product was present in higher concentration than obtained. As the recrystallization process has not been optimized, there are probably margins for improvement. The NMR spectrum of $MeRe(BAV)_2$ (**74**) is shown in Figure 3.1 together with peak assignments. The numbers and letters on the spectra refer to the molecule above.

Most of the aromatic signals (6.5-8 ppm, 100-160 ppm) have not been assigned due to overlapping. Even if the resolution allowed proper assignment, most of the aromatics will be eliminated in the next step of the synthesis, after deprotection. Some minor impurities are present in the sample. Protons 4 and 5 have been distinguished even if chemically equivalent in anticipation of the next synthetic step. Protons 2 and 3 appear to be equivalent in this molecule, even if expected otherwise. This phenomenon is discussed later (p.89)

a)

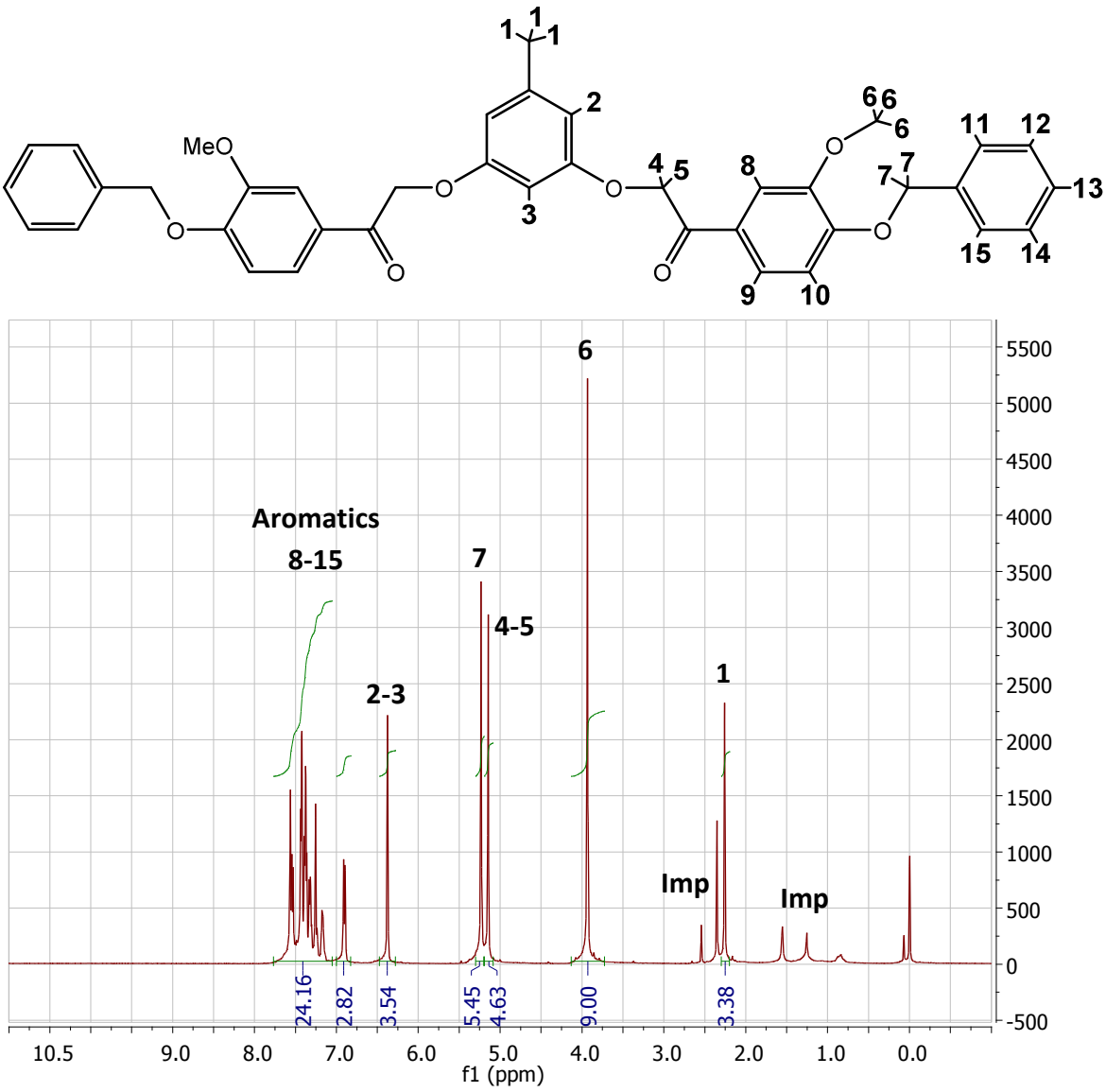


Figure 3.1 a) ^{1}H NMR spectrum of MeRe(BAV)$_2$ in$CDCl_3$+1%TMS

b)

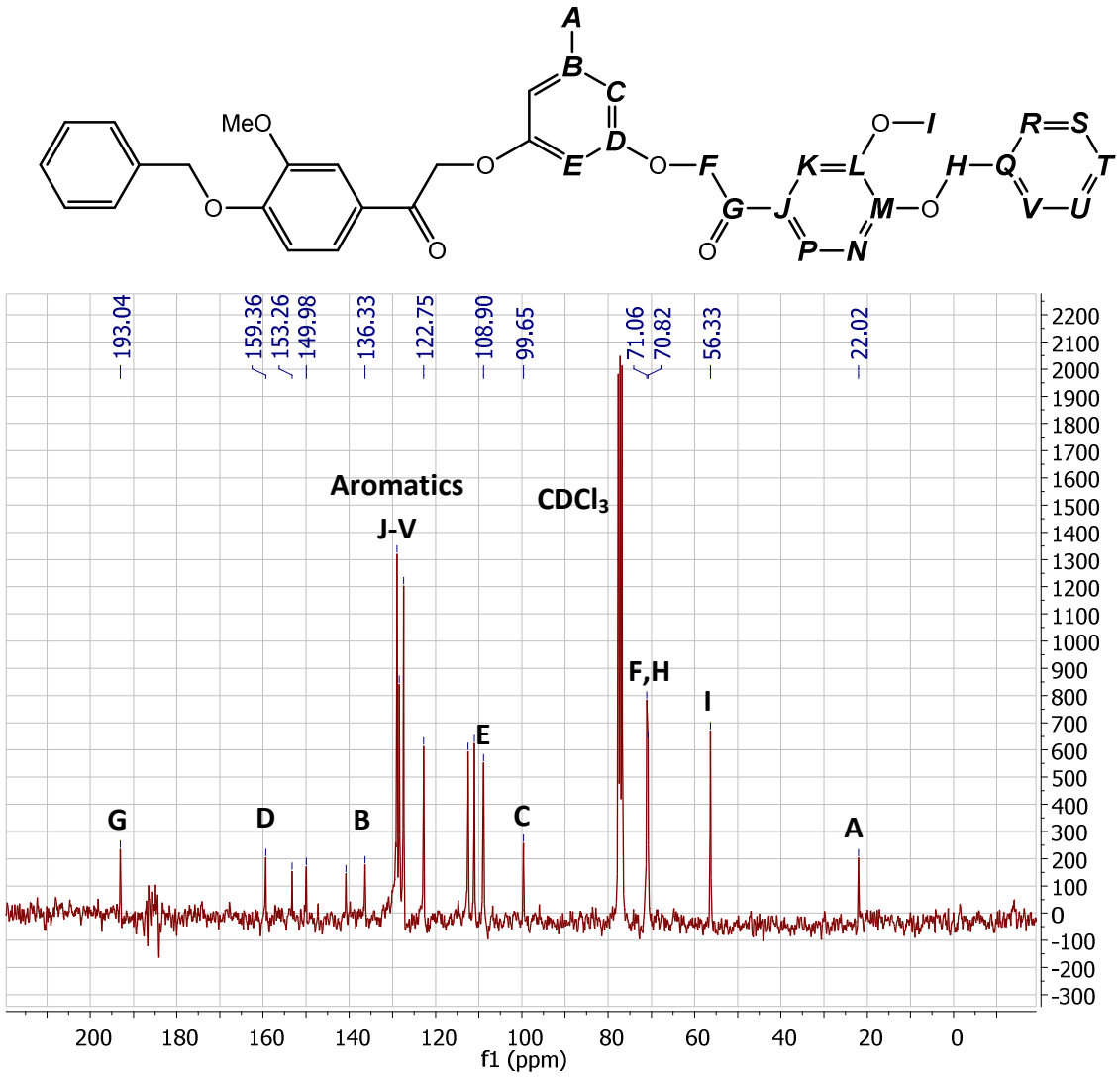


Figure 3.1 b) ^{13}C NMR spectrum of $MeRe(BAV)_2$ in$CDCl_3$+1%TMS

Step 2: hydrogenation

Hydrogenation was performed with two goals, deprotecting the phenols from the benzyl group and reducing the ketones to alcohols. This is why the reaction conditions are harsher, compared to standard benzylic deprotection procedures. In the first attempts EtOH and THF were employed as solvents without success, while reaction in DMF yielded ≈95% pure MC1 (**75**). The precise yield was hard to measure because some solvent was always left in the sample, but the selectivity and conversion of this reaction step were very high. The NMR spectrum of MC1 is shown in Figure 3.2 together with peak assignments. The numbers and letters on the spectra refer to the molecule above.

a)

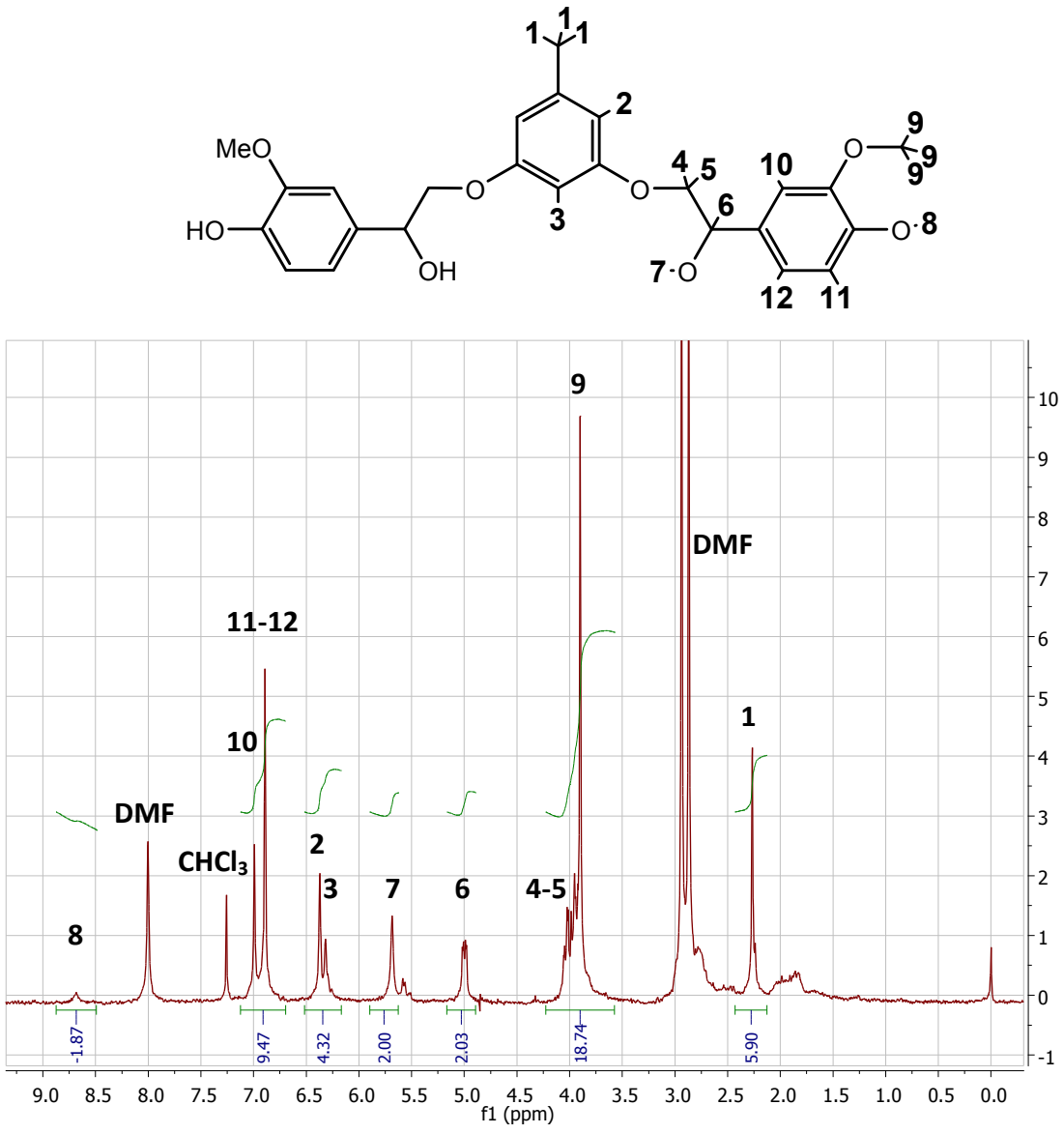
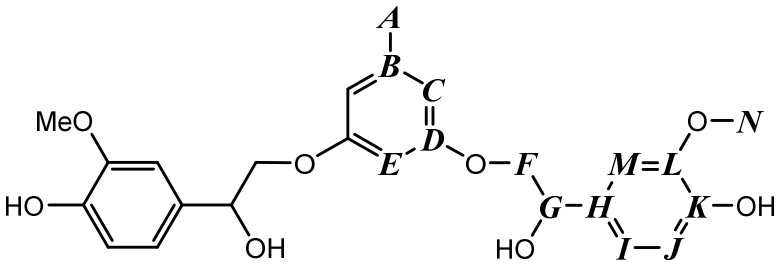


Figure 3.2 a) ^{1}H NMR spectrum of MC1 in $CDCl_3$+1%TMS

b)



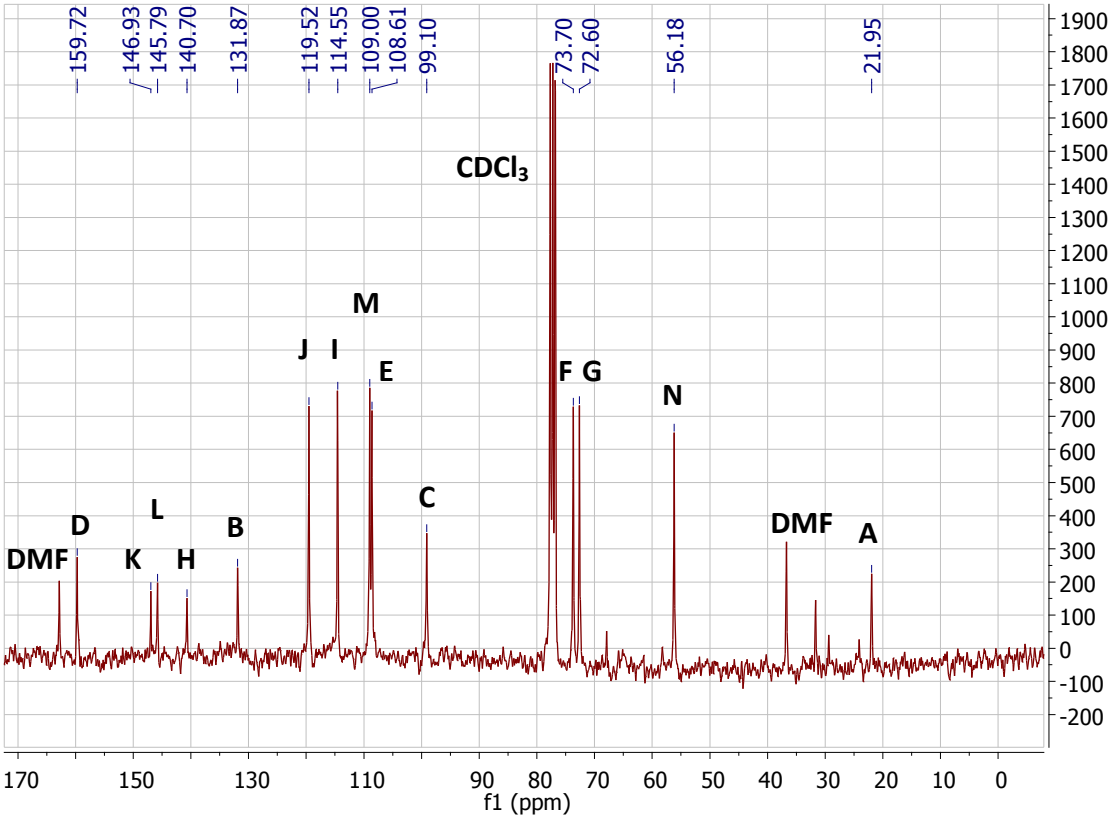


Figure 3.2 b) ^{13}C NMR spectrum of MC1 in$CDCl_3$+1%TMS.

DMF was never completely evaporated from the sample. Protons 2 and 3 are now non equivalent as expected, as well as protons 4 and 5.

Considerations

There is not much to be said about this synthesis, except that it worked, and that good results were achieved. The only problem of this reaction was the recrystallization process. It needs to be optimized or purification after step 1 needs to be otherwise improved.

3.1.2 MC2

The reaction scheme for MC2 (**78**) is presented in Figure 2.2 (p. 70). The main obstacle for this synthesis was the low solubility of MC2 and its precursors. The reaction was further complicated by a misinterpretation of the data, which will be discussed.

Step 1: nucleophilic substitution

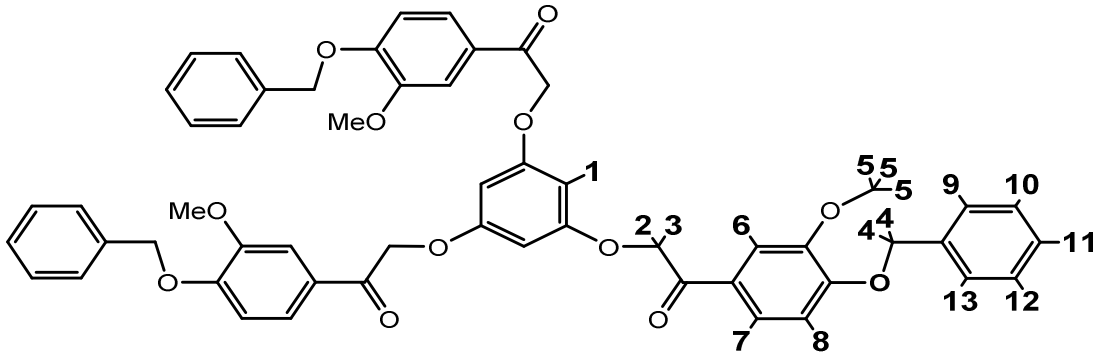
The first attempt for this synthesis was conducted at 40°C using KOH as base, as described in the previous chapter (p.70 section 2.2.2). The reaction produced both, a liquid and a solid phase, with 46.5% and 21.3% crude yield, respectively. Analysis of the liquid phase revealed the presence of $Ph(OBAVone)_3$ (**77**), our target molecule, together with several other impurities. Attempts of purification were made by silica column chromatography and Biotage separation, with several eluents. The results obtained were not encouraging, with low purification yield and incomplete purification. Meanwhile the solid fraction was analyzed. The solid was insoluble in most solvents, and only slightly dissolved in the others. The IR analysis didn't give any useful information, therefore a small amount of the sample (<1mg) was partially dissolved in DMSO D_6 to record a NMR spectrum. The results were surprisingly good, apparently the solid fraction was composed by pure $Ph(OBAVone)_3$. The NMR spectrum is reported in Figure 3.3, together with peak assignments. The numbers and letters on the spectra refer to the molecule above. The same consideration about aromatic signals and equivalent protons made for $MeRe(BAV)_2$ (**74**) are valid for $Ph(OBAVone)_3$.

The next step was naturally to optimize the reaction to increase the yield of the solid product. Table 3.2 reports the most significant attempts and their reaction conditions. The conditions used in attempt n° 4 produced satisfactory results.

Attempt n°	Base employed	Base addition conditions	Temperature (°C)	Liquid crude yield (%)	Solid crude yield (%)
1	KOH	Slow addition	40	46.5	21.3
2	K_2CO_3	Excess (130%)	40	38.8	29.7
3	K_2CO_3	Excess (130%)	90	43.3	26.3
4	K_2CO_3	Excess (130%)	25	Discarded	51.2
5	KOH	Slow addition	25	71.9	13.1

Table 3.2 resumes the reaction conditions, and the relative yields, tested for the nucleophilic substitution step of MC2 synthesis.

a)



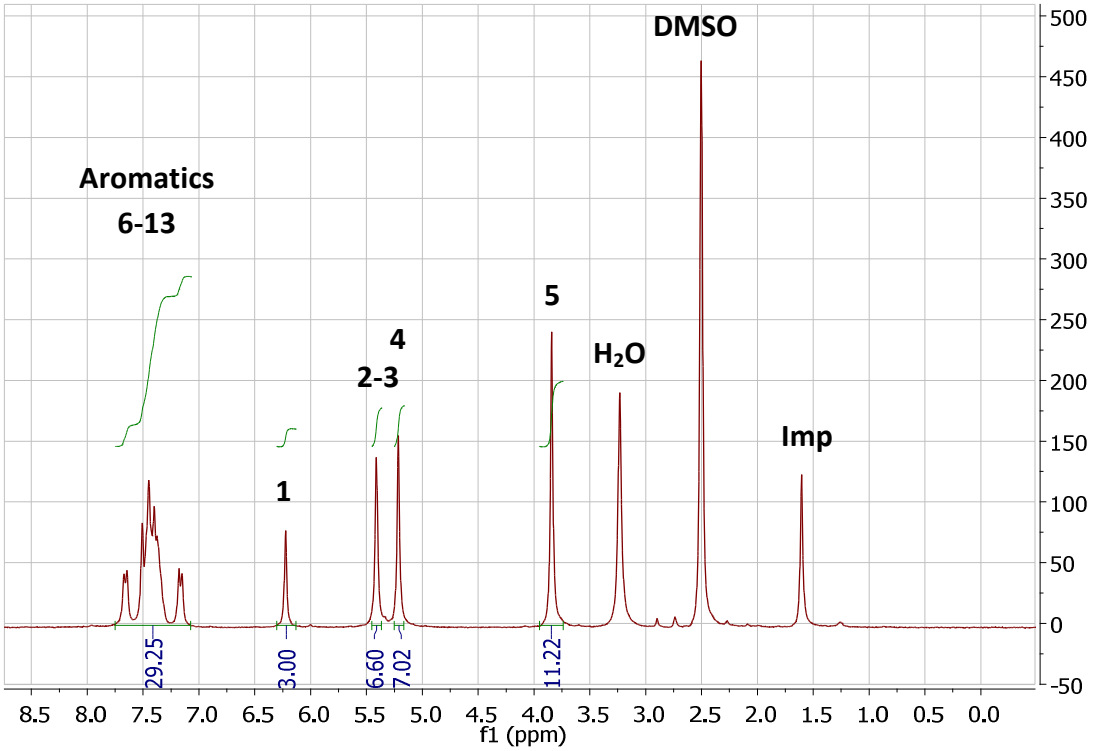


Figure 3.3 a) ^1H NMR spectrum of $Ph(OBAVone)_3$ in DMSO

b)

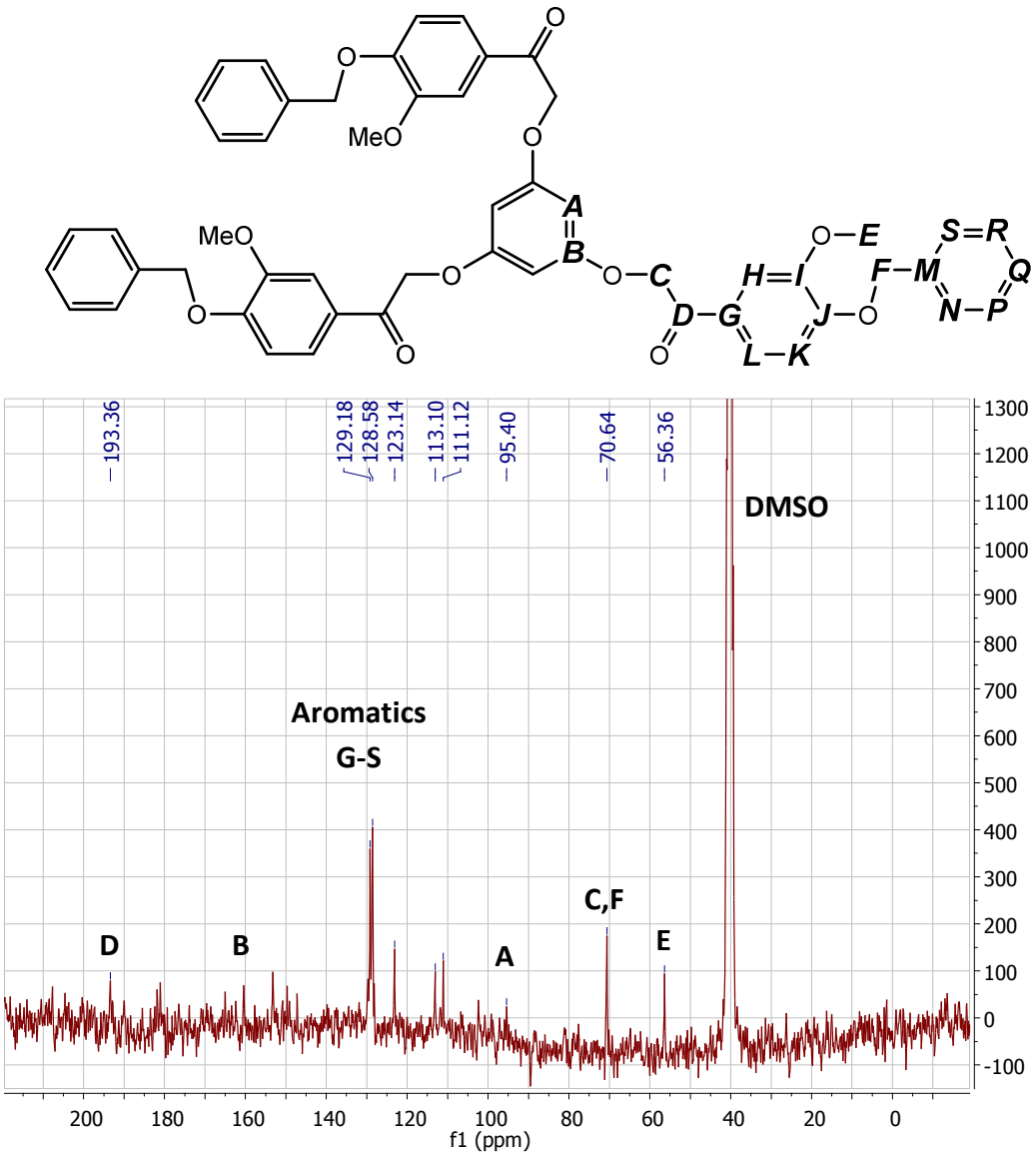


Figure 3.3 b) ^{1}H NMR spectrum of Ph(OBAVone)$_3$ in DMSO. The low solubility is responsible for the poor quality of the spectra, expecially the ^{13}C.

Step 2: hydrogenation

The low solubility of Ph(OBAVone)3 (**77**) has been an issue in its hydrogenation. THF was used as solvent because it partially dissolved our solid. The hydrogenation still had to be performed on a suspension of both the catalyst and Ph(OBAVone)3, with only small amounts of reactant in solution. Harsh reaction conditions were needed to reduce the carbonyls to alcohols. The raw product of the hydrogenation was an oily mixture, partially soluble in most solvents but hardly completely soluble. It was impossible to measure the crude yield, despite several attempts relevant amounts of solvent always remained in the

sample. The product mixture was apparently composed by an aromatic fraction, most likely our product, and an aliphatic impurity. After several tries (organic solvents washing, extractions and precipitation) the purification procedure described in the previous chapter (p. 71 section 2.2.2) was perfected, and pure MC2 (**78**) was obtained with a yield of 33.5%. The NMR spectrum is reported in Figure 3.4, together with peak assignments. The numbers and letters on the spectra refer to the molecule above. Protons 2 and 3 still appear to be equivalent, despite expectation. This phenomenon is discussed below (p. 89)

a)

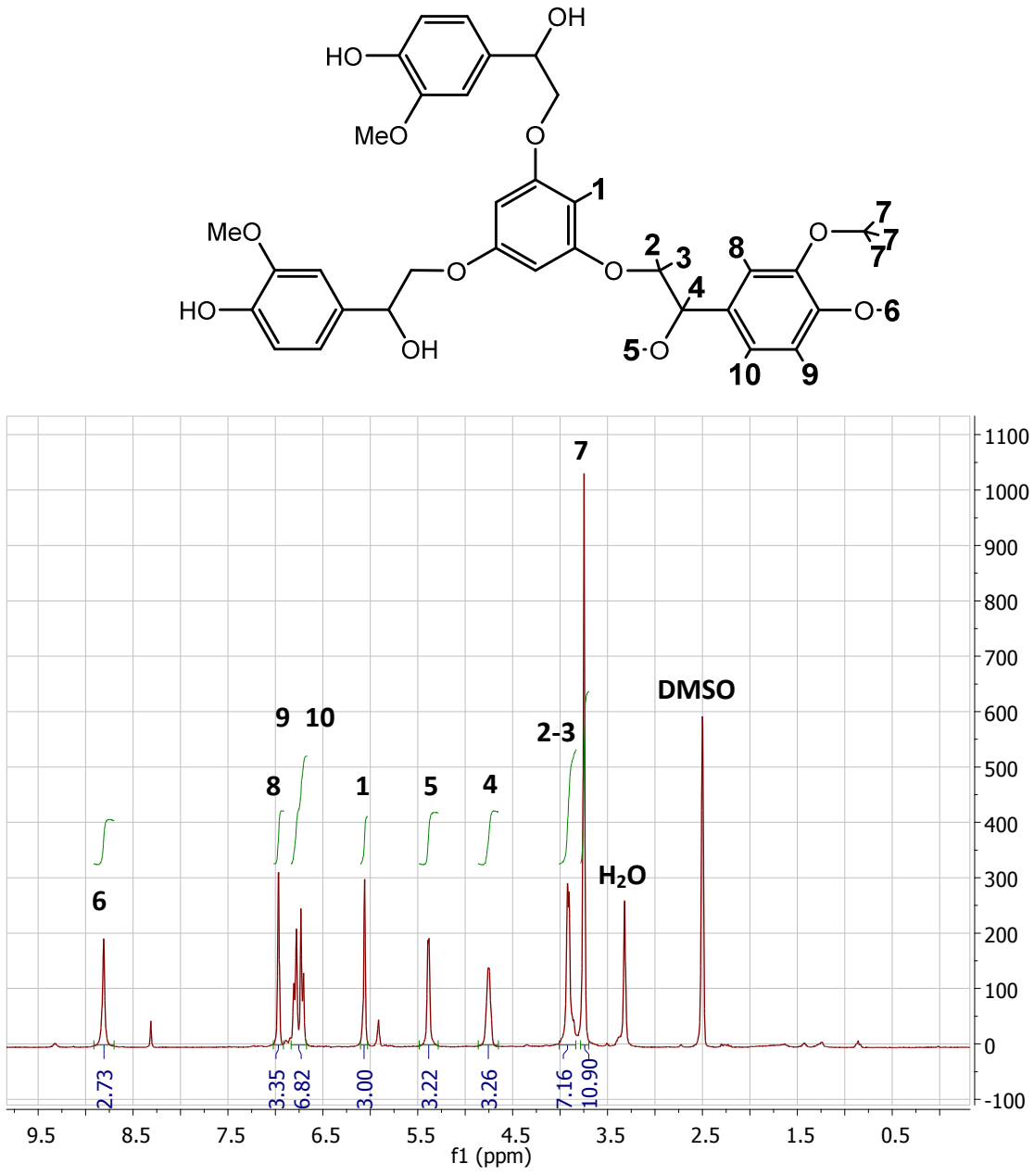


Figure 3.4 a) ^{1}H NMR spectrum of MC2 *in DMSO.*

b)

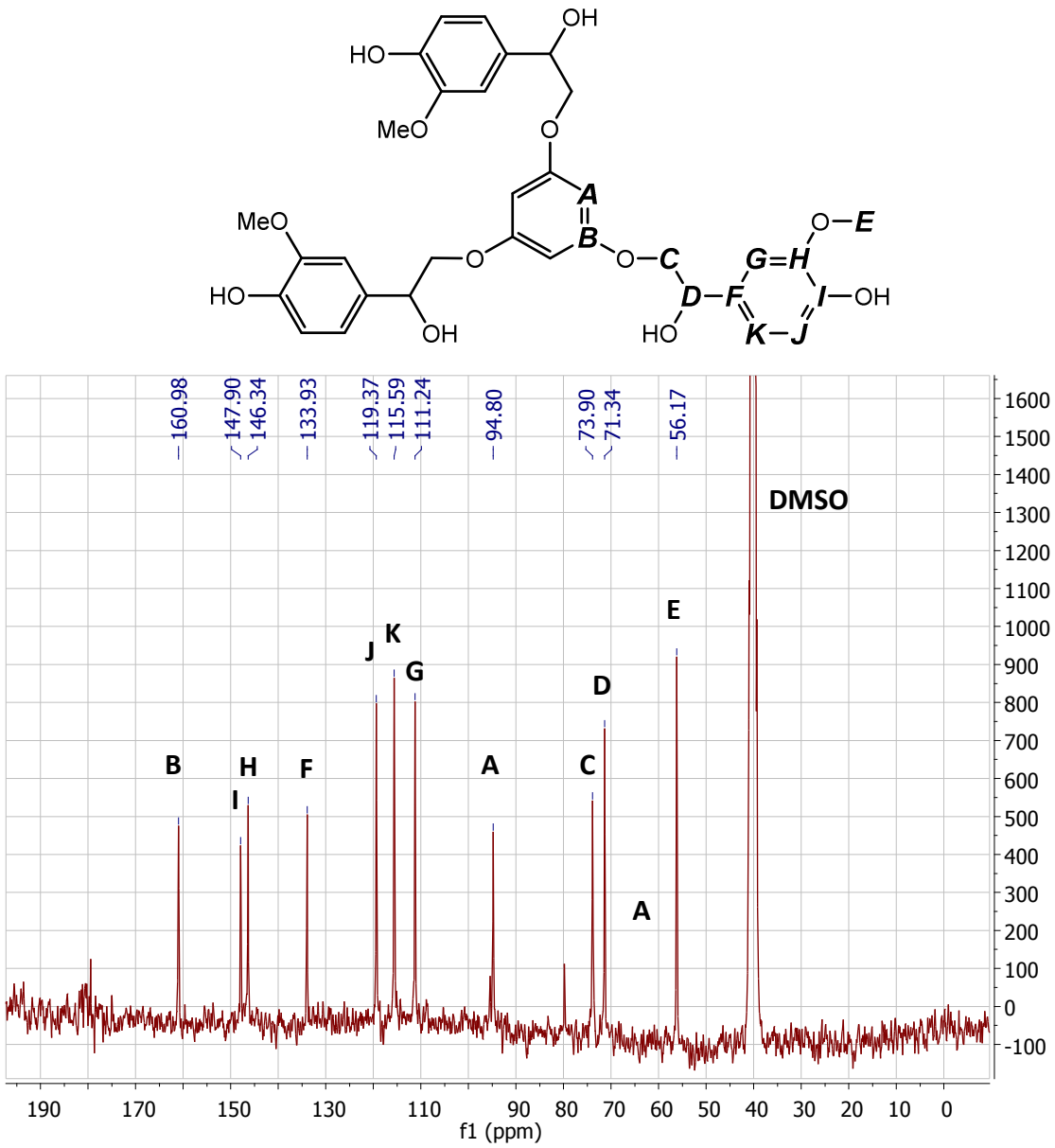


Figure 3.4 b) ^{1}H *NMR spectrum of* MC2 *in DMSO.*

The high amount of impurity present in the hydrogenation product already hinted that something was not going as expected. The final confirmation came when the new samples were hydrogenated. The first Ph(OBAVone)$_3$ employed as reactant was synthesized using reaction conditions n° 1 (KOH, 40°C) while the new samples came from reaction n°4 (K_2CO_3, RT). The NMR spectra of both samples was identical, the product of both reactions appeared to be pure Ph(OBAVone)$_3$. Despite this apparent similarity, hydrogenation performed with the new samples never produced MC2, even using different reaction conditions.

MC2 synthesis: misinterpretation

The data provided by hydrogenation of the K_2CO_3 synthesized solid, led us to formulate a hypothesis. Unfortunately, it has not been confirmed experimentally for lack of time. We believe that $Ph(OBAVone)_3$(**77**), the target molecule of the nucleophilic substitution step, is mostly found in the liquid fraction, together with other impurities in significant amount. During the reaction, a fraction of $Ph(OBAVone)_3$ is trapped in the solid phase. The structure of the solid phase is unknown, but it is most likely an insoluble polymer. Apparently, the other impurities present in the liquid phase are not trapped in the solid phase. The difference between K_2CO_3 and KOH reactions is $Ph(OBAVone)_3$ concentration. In the first, a high amount of solid polymer is produced, which traps the low quantity of $Ph(OBAVone)_3$ present in solution. In the second, the small amount of solid produced is saturated with the target molecule. The NMR spectra of the two compounds are identical; since the solid is insoluble, the only thing that gets analyzed is the $Ph(OBAVone)_3$ trapped inside. When the product is hydrogenated, the difference becomes clear. In the KOH case, $Ph(OBAVone)_3$ is present in high concentration in the solid (hypothesis: 40%). During hydrogenation both the solid polymer and $Ph(OBAVone)_3$ react, yielding MC2 (**78**) and the aliphatic impurities as product. MC2 can be then purified and gathered. In the K_2CO_3 case, the concentration of $Ph(OBAVone)_3$ is too low (hypothesis 1-5%) and hydrogenation yields almost exclusively aliphatic impurities. This hypothesis fits the experimental data, but it has to be proven experimentally. To resume:

1. nucleophilic substitution reaction (step 1) with both K_2CO_3 and KOH produces a liquid and a solid fraction. The yields of the two fractions are considerably different and most likely their composition as well.
2. $Ph(OBAVone)_3$ appears to be present in the liquid fraction, but the purification is complicated.
3. The NMR spectra of the solid fraction from both reactions are identical, and appear to be pure $Ph(OBAVone)_3$.
4. Hydrogenation reaction performed on the solid obtained from KOH reaction produces MC2, hydrogenation on K_2CO_3 solid does not.
5. Our hypothesis is that the solid is an insoluble polymer, which somehow traps $Ph(OBAVone)_3$, and reacts with H_2 to produce an aliphatic impurity.

Considerations

The target molecule MC2 (**78**) has been synthesized, which is a good result, but the overall 10% yield is a major drawback of this synthesis. Unfortunately most of the work devoted to this synthesis was useless, because of the solid fraction data misinterpretation. Most likely, there are many chances to significantly improve the reaction yield, once the correct parameters will be optimized. To this end some preliminary experiments have been performed. For example attempt n° 5 (Table 3.2 p.84), significantly increased the liquid fraction yield. Reaction of $Ph(OBAVone)_3$ (**77**) with even stronger bases might further improve the process.

MC1 and MC2 comparison

The aim of this paragraph is to discuss an interesting difference between MC1 (**75**) and MC2 (**78**), which should be, in principle, very similar. Figure 3.5 shows the H^1 spectra of both compounds, between 3.5 and 4.5 ppm. The interested part of the molecule is nearly identical in both molecules, and yet a difference in the two spectra is observable. The ≈0.2 ppm difference between the two samples can be explained by the different solvents, $CDCl_3$ for MC1 and deuterated DMSO for MC2. The interesting part is the difference in the resonance pattern of protons 4, 5 and 6 for MC1 and 2, 3 and 4 for MC2. MC1 behaves as expected, the chiral center on carbon G makes the two protons on carbon F magnetically different. Therefore, as expected, a doublet of doublets is present at 5 ppm, generated by proton 6 coupling with the both proton 4 and 5. Three distinct signals are present at 4 ppm, the one at lower ppm belongs to the methyl protons 9 and is not interesting. The two remaining signals form a multiplet, consistent with two overlapping doublets of doublets. Each proton, 4 and 5, couples with the other and with proton 6, theoretically producing 8 signals. The assignment is confirmed by 2D spectra. On the other hand, the spectrum produced by MC2 is much simpler. Despite the presence of the same chiral center, on the same carbon, protons 2 and 3 behave as magnetically equivalent. In fact only two doublets are visible in the spectrum, one at 4.7 ppm and one of double intensity at 3.8 ppm. They are originated by the single proton 4 and the two equivalent protons 2 and 3, coupling with each other.

It is possible that some steric effects are responsible for this anomaly, but we don't have any convincing explanation for this phenomenon. Only another unusual behavior displayed by those molecules can be offered. Methylresorcinol (**72**) H^1 spectrum shows only 3 peaks, one for the two alcoholic groups, one for the three methyl protons and one for the three

aromatics. This is surprising because the three aromatic protons are supposedly non equivalent, as the substituents on the aromatic ring are different. This apparent identity vanishes as soon as the alcoholic groups are derivatized. For example, when MeRe is acetylated, the NMR spectrum displays two aromatic peaks, with intensity 2:1 as expected. Figure 3.6 shows the NMR spectra of MeRe and acetylated MeRe, with peak assignments. A similar phenomenon happens when a molecule of MeRe(BAV)$_2$ (**74**) is converted to MC1 (**75**), as can be seen by comparing Figures 3.1 and 3.2

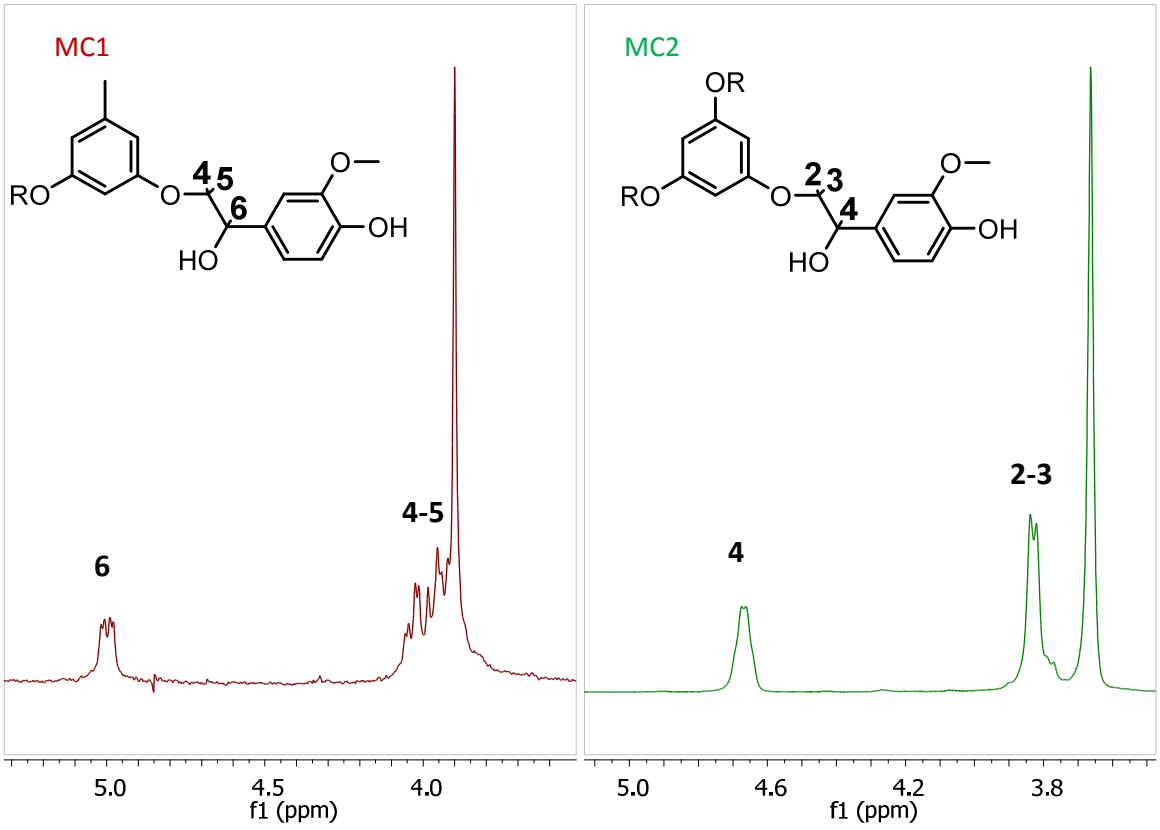


Figure 3.5 compares the ^1H spectra of the two model compounds MC1 and MC2 ($CDCl_3$ + 1%TMS and DMSO, respectively). As can be seen, the peak multiplicity is different in the two cases, even if the molecules are very similar. MC1 spectrum is consistent with our expectation, on the other hand protons 2 and 3 appear to be chemically equivalent in MC2.

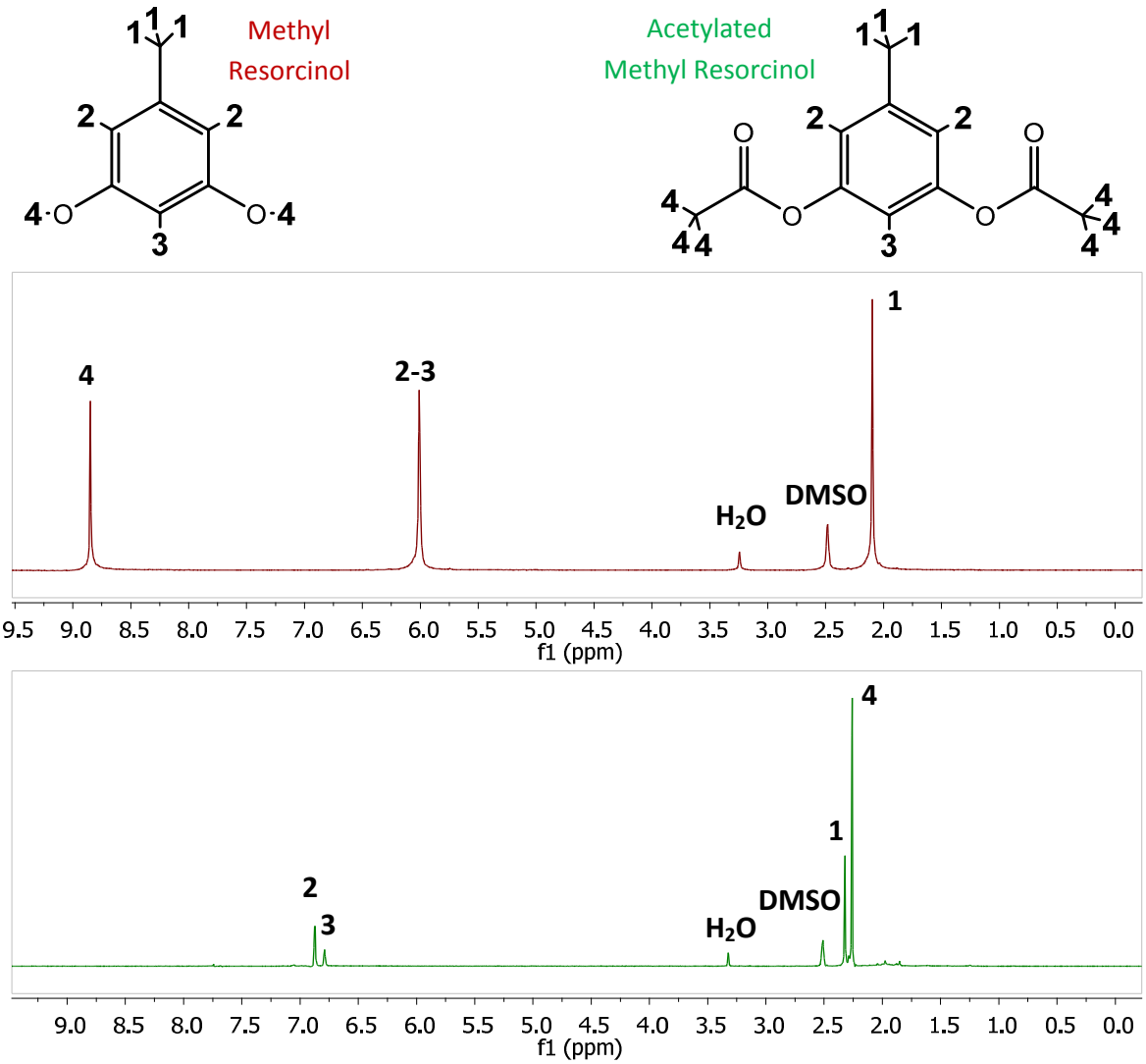


Figure 3.6 shows a comparison between 5-methylresorcinol and acetylated 5-methylresorcinol ^{1}H spectra in DMSO. It is important to notice the change of pattern of the aromatic protons 2 and 3.

3.1.3 MC3

The reaction scheme for MC3 synthesis is presented in Figure 2.3 (p. 72). This was the most complicated and most similar to lignin of the synthesized model compounds. A synthesis pathway completely different from the other two was adopted, but unfortunately it didn't produce good results.

Benzyl vanillin synthesis

There is not much to say about this synthesis. As reported in the Experimental chapter (p. 73 section 2.2.3) an optimized procedure for the process was available. All the benzyl vanillin (**79**) required for the project was synthesized in a single attempt, without problems. The overall yield was 79.2%.

Step 1: nucleophilic substitution

The first step of this reaction did not present any particular problem. The reaction was performed since the first attempt as described in the previous chapter (p. 73 section 2.2.3). The desired product, triester (**83**), was obtained with a yield of 46.8% on the best attempt. The NMR spectrum is reported in Figure 3.7, together with peak assignments. The numbers and letters present of the spectra refer to the molecule above.

a)

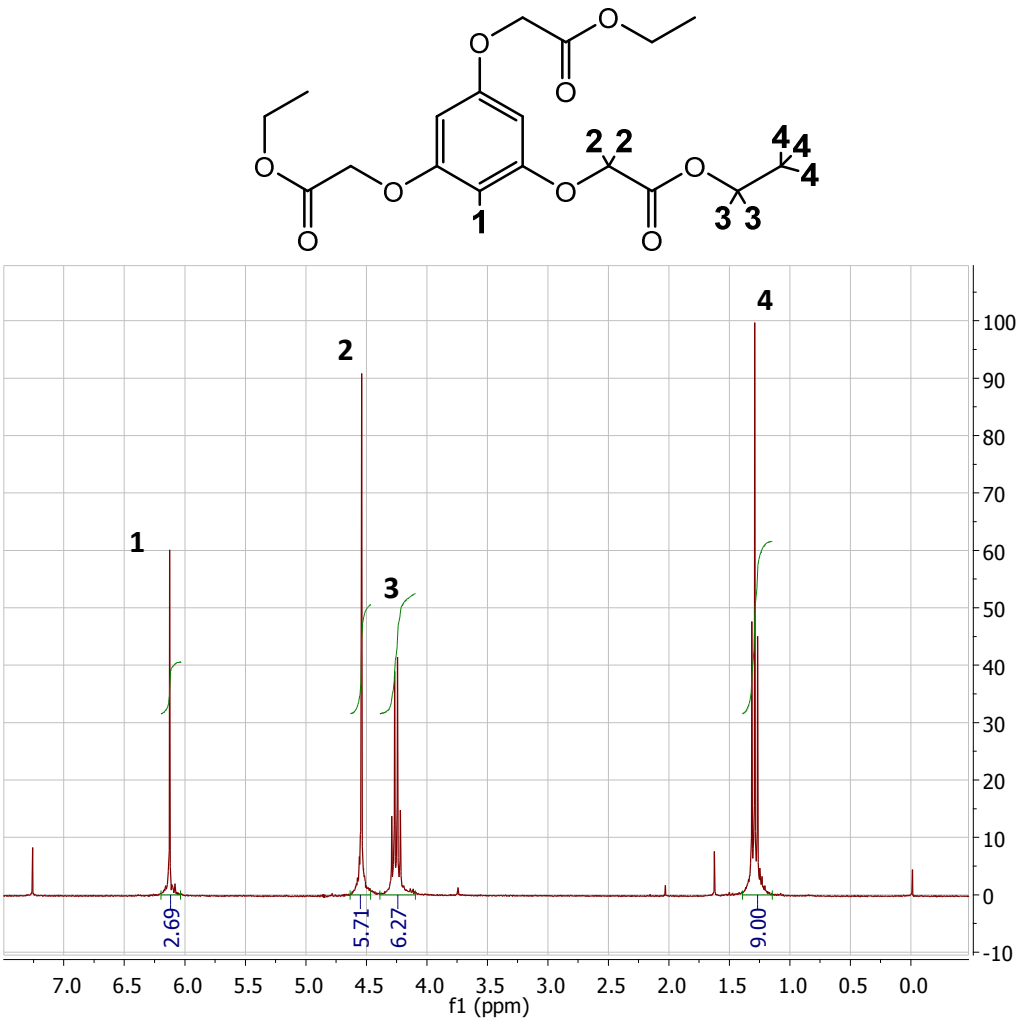


Figure 3.7 a) ^{1}H NMR spectrum of triester in $CDCl_3$+1%TMS.

b)

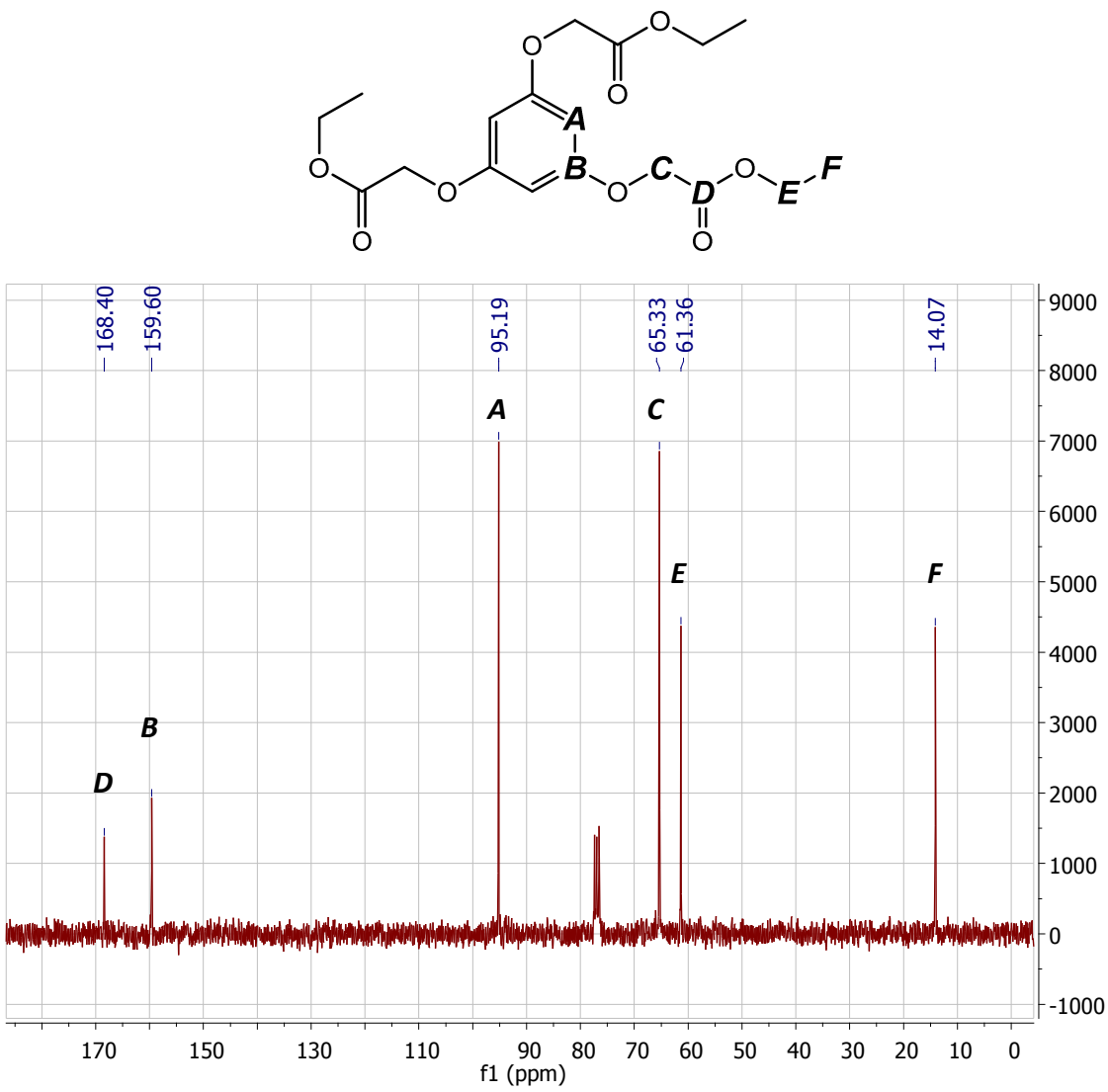


Figure 3.7 b) ^{13}C NMR spectrum of triester in $CDCl_3$+1%TMS.

Step 2: aldol condensation

The condensation step is where this synthesis stopped. In fact, despite several attempts, reasonable yields or proper purification were never achieved. The problem of this reaction is most likely related to its high steric hindrance. The reaction conditions of the most relevant attempts are reported in table 3.3. The only changing parameters are temperature and reaction time, everything else remained constant, as described in the experimental chapter (p. 73 section 2.2.3). To ensure complete conversion, benzyl vanillin was in slight excess over the amount of trimester, BuLi was in excess over benzyl vanillin and DIPA was in excess over BuLi.

Attempt n°	Reaction time (h)	Temperature (°C)	Crude yield	Pure yield
1	3	-70	45%	1.7%
2	20	-70 to RT	52%	0%
3	10	-70	77%	3.8%
4	2	-70	71%	0.69%

Table 3.3 resumes the reaction conditions tested and the obtained yields for the condensation step for molecule MC3.

Purification with column chromatography was performed. The mixture toluene/EtOAc 2:1 was tested among others and chosen as the best eluent. Unfortunately the purification was very hard to achieve, the reaction produced a high number of similar molecules, hardly separable. A perfectly pure tricondensate (**84**) sample was never obtained, and the yields reported in table 3.3 refer to only partially pure samples. The purity of the samples was enough to identify and characterize the target molecule, but the obtained spectra clearly show the presence of impurities. The NMR spectrum is reported in Figure 3.8, together with peak assignments. The number and letters on the spectra refer to the molecules above. The aromatic region was too crowded for proper peak assignment.

a)

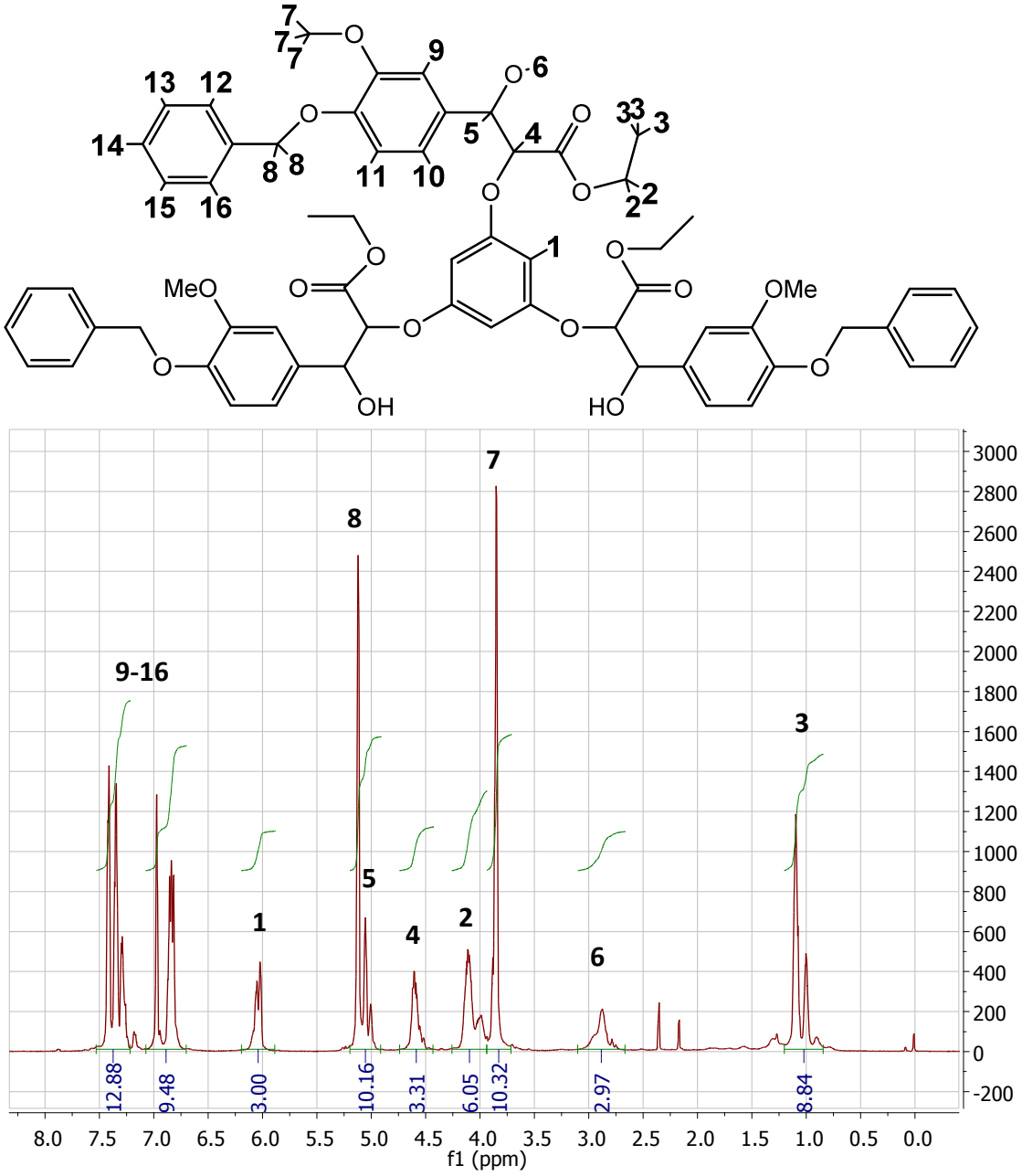


Figure 3.8 a) ^{1}H NMR spectrum of tricondensate in $CDCl_3$+1%TMS.

b)

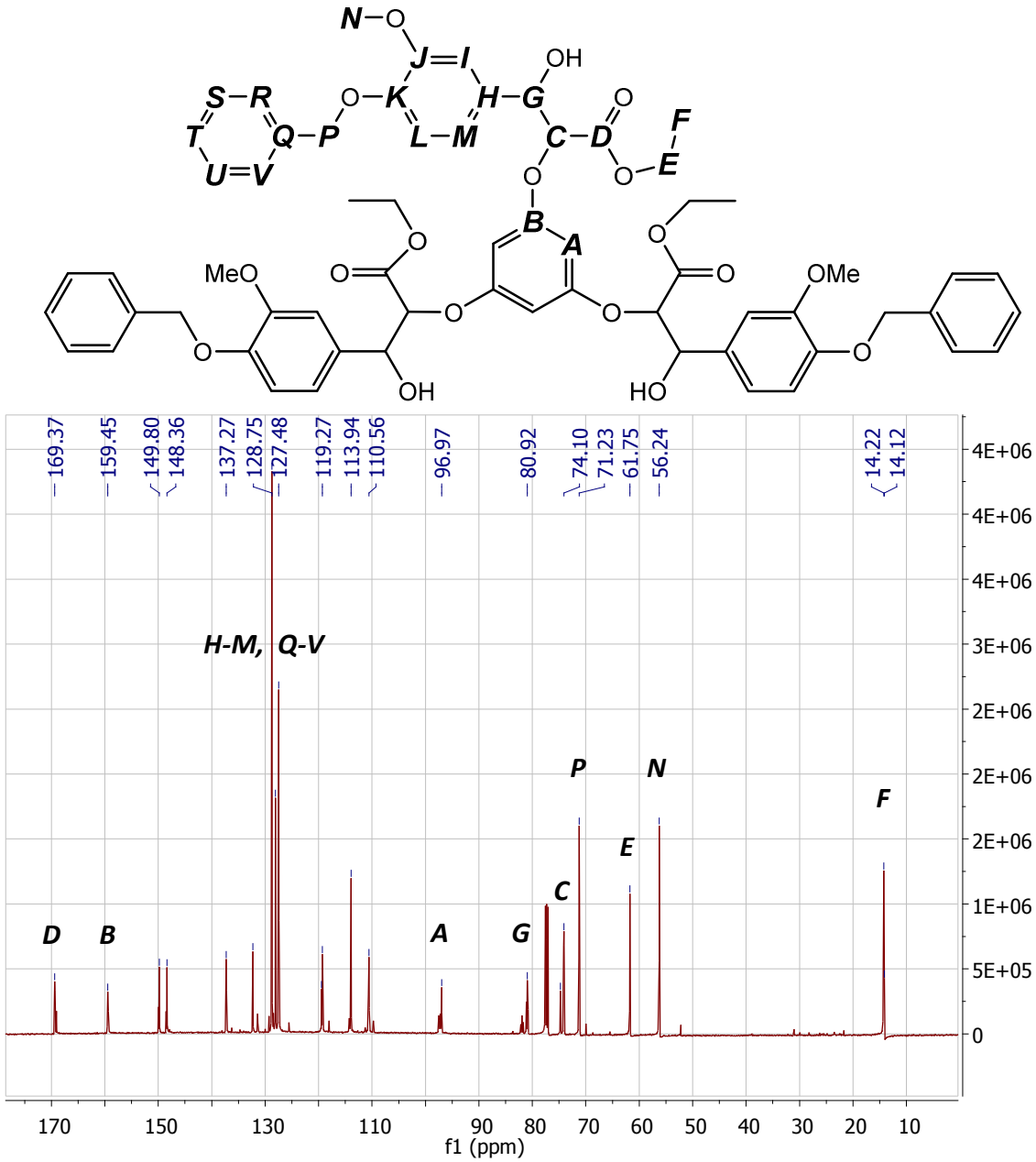


Figure 3.8 b) ^{13}C NMR spectrum of tricondensate in $CDCl_3$+1%TMS.

The shape of the peaks clearly indicates the presence of several molecules, similar but different. Possibly enantiomers.

As can be seen from table 3.3 the amount of product obtained was too low, and since this synthetic route did not look promising, the synthesis has been abandoned. It is still possible to draw some conclusion from the results obtained. Attempts to increase the yield by increasing the temperature failed, as shown by attempt n° 2. After a couple of hours at -70°C the reaction temperature was allowed to rise to RT, and the reaction left overnight. This attempt did not generate the desired product, which suggests that the reaction has to be left at -70°C for its entire course. By comparing attempts 1, 3 and 4, and considering the amount of unreacted benzyl vanillin (**79**) found in each experiment, it is clear that the reaction time is crucial for the reaction yield, but since the yield remained extremely low even after 10h of reaction the process was considered too expensive and unproductive, and the synthesis abandoned. It is to be said that with such low yields, the small amounts of material involved and the hard purification, the data reported might not be accurate.

Considerations

The synthesis of MC3 (**71**) and its polymerization study were more or less the main objective of our project in the beginning. The difficult synthesis convinced us to focus on easier molecules first, and possibly return on MC3 later on. The synthesis of MC3 needs to be improved, but it is hard to tell if it is better to optimize the reaction proposed or to try new synthetic routes. For the first hypothesis, the most obvious attempt is to increase even more the reaction time. To do that it would be necessary either to add dry ice to the ethanol bath every few hours for days, or to find a better way to cool the system at -70°C. As a more suitable alternative, a new synthetic route could be based on MC2 (**78**) synthesis. $Ph(OBAVone)_3$ (**77**) is the product of the first nucleophilic addition step, and could be considered as the starting point of the new synthesis. The protons in α to the carbonyl are reactive, and can be removed with a strong base. The carbanion could then be reacted with protected chloromethanol, to introduce the missing methanol in the molecule. The only difference between MC2 and MC3 is the extra carbon and hydroxyl group that could be added with chloromethanol. Therefore, after reduction of the carbonyl and deprotection, MC3 could be obtained. MC3 is the more similar to lignin of the three proposed model compounds and it would be very important to test its reactivity during polymerization. Future research might need to find a proper way to synthesize MC3, if the study of the other molecules will give encouraging results.

3.1.4 CONCLUDING REMARKS

To summarize all what was accomplished in a few lines, the synthesis of two of the three target lignin model compounds was accomplished. The synthesis of the first and easiest of the molecules, MC1 (**75**), was achieved without major troubles and acceptable yield, only one problem remains on a purification step. The second molecule, MC2 (**78**), was obtained, purified and characterized. The reaction yield was too low to study MC2 polymerization, but a proper optimization will probably give good results. It is important to mention that this was the first synthesized tridentate model compound for lignin. MC3 (**71**) was the most complicated and similar to lignin of the proposed model compounds, and unfortunately its synthesis was never achieved. A different synthetic route, compared to the other two, was tested, but despite the several attempts, the purification after the condensation step was never satisfactory. Some alternative routes to obtain MC3 have been proposed, as well as some possible developments of future work.

3.2 Polymerization

The study of DHP polymerization was the initial purpose of our research. Coniferyl alcohol (**29**) was polymerized in presence of another molecule, the lignin model compound. The aim was to analyze the obtained DHP, in order to understand the effects of the MC on coniferyl alcohol polymerization. Our hypothesis, proposed in the introduction (p. 61 section 1.4.1), is that using a tridentate MC, the presence of coniferyl alcohol "closed loops" in DHP will be increased. Hopefully it will be possible to identify and measure them; and once this is accomplished, to detect their presence, or lack of it, also in natural lignin. Positive results might be helpful for the understanding of the mechanisms of lignin biosynthesis. On the more practical side, a working polymerization between our MCs and coniferyl alcohol might have some useful applications. The starting aim of our research was to perform CA polymerization with the three MCs described previously (MC1 (**75**), MC2 (**78**), MC3 (**71**)). Unfortunately only MC1 was available for the polymerization study, therefore MC1, 5-methylresorcinol (**72**) and phloroglucinol (**76**) were used as central molecules (CMs). Even without using MC2 and MC3 as central molecules, this study can lead to useful results. MeRe and $Ph(OH)_3$ are the precursors of our synthesized MCs and therefore they will provide useful information and background

for the future analysis of our model compounds. Even if their structure is not similar to lignin, they will most likely couple with coniferyl alcohol. And since their geometry is the same as the MCs, hopefully they will have similar effects on lignin polymerization. Investigation of MeRe and $Ph(OH)_3$ DHP has to be considered as a preliminary study for the research on the synthesized MCs.

General considerations

Most of the time, the polymerization reaction yielded both a solid and a liquid fraction, the heavy and light fraction of DHP, respectively. The light fraction was extracted from the reaction mixture by an organic solvent, while the heavy one was simply filtered out and washed. In order to obtain a closed loop, a good number of CA molecules have to be connected to the CM, forming a molecule with a high Mw. Because of the increased weight and the low solubility of our molecules, the product of interest is more likely found in the heavy fraction. On the other hand, the first signs of the closed loops formation, i.e. CM-CA cross-coupling product, will be found in the light fraction. It was decided to focus on the light fraction for two reasons. The first is that we don't know what kind of signal is produced by the "closed loops", the second is that the heavy fraction is more complicate to analyze. The higher Mw of the heavy fraction causes both, a lower solubility and a lower analysis resolution, especially for NMR. Analysis of a high Mw DHP sample produces a low intensity and unresolved NMR spectrum. Starting the study from the light fraction is the solution to both problems: the analysis is clearer and more interpretable, making it easier to identify the traces of CM-CA cross-coupling. Using these traces as starting point it will be possible to detect the "closed loops". Analysis of the light fraction will provide the background information necessary to analyze the heavy fraction and achieve our goal. This is why the next paragraphs will be focused on light fraction analysis and CM-CA cross coupling, even if the initial aim of this research was the discovery of "closed loops".

In order to produce a wide NMR database and increase our chances to detect the unknown signal of closed loops and CM-CA cross coupling, it was decided to proceed progressively, by steps. Each CM was polymerized with gradually increasing amount of coniferyl alcohol. As reported in table 3.4, each CM was first reacted without coniferyl alcohol, to make a blank reference. Then the CMs were reacted with CA in a 1:1 ratio, followed by a1:1 and 1:2 CM free alcohols/CA polymerization. Coniferyl alcohol without any CM was polymerized to produce a normal DHP. This sample was then used as a reference for the detection of CM-CA cross coupling. Interactions between CMs and CA are different from

CA-CA interaction, and produce a distinct signal in the ^{13}C NMR spectrum. After analysis of the blank DHP spectra and assignment of as many peak as possible, the normal DHP spectra were matched against the CM DHP's ones, looking for differences. Those differences were then further studied, to understand if they are originated by interactions between CM and CA. Our research was based on NMR and SEC analysis, the two available methods most suited for the kind of work. SEC provides immediate and simple information about the samples mass, while NMR provided more precise structural information. Some of the heavy fractions were not analyzed by SEC for solubility issues. HPLC was performed on most of the samples, but when used for simple comparison its results were similar to SEC's. Of course if HPLC was used as a separation technique, and each fraction analyzed, it would provide much more useful information, but this research is at a too early stage to justify such a long procedure. It is better to rely on simpler analysis until more encouraging results are found. Table 3.4 resumes the performed experiments, together with some relevant information.

3.2.1 CONIFERYL ALCOHOL

As said before, the pure coniferyl alcohol DHP, synthesized without CM, is the base for our data interpretation. It will be analyzed in detail. A polymerization of 200mg of coniferyl alcohol (**29**) yielded 33 mg of heavy and 83 mg of light fraction. The overall yield, calculated using the reactant and product masses, is 58%, with the light and heavy fractions yield being 41.5% and 16.5%, respectively. Those fractions were acetylated and analyzed, according to the procedure described in the experimental chapter (p. 75)

Sample Name	Central Molecule	CM/CA (mol/mol)	Acetylation	NMR solvent	SEC	HPLC	Conditions
Ul_MR_p01_L	MeRe	/	Yes	DMSO	Yes	Yes	No CA
Ul_MR_p02_L	MeRe	1:1	Yes	acetone	Yes	x	
Ul_MR_p02_H	MeRe	1:1	Yes	acetone	x	x	
Ul_MR_p03_L	MeRe	1:2	Yes	acetone	Yes	Yes	
Ul_MR_p03_H	MeRe	1:2	Yes	acetone	Yes	Yes	
Ul_MR_p06_L	MeRe	1:2	No	DMSO	x	x	pH 4
Ul_MR_p07_L	MeRe	1:2	Yes	$CDCl_3$	Yes	Yes	pH 4
Ul_MR_p07_H	MeRe	1:2	Yes	DMSO	Yes	Yes	pH 4
Ul_MR_p05_L	MeRe	1:4	Yes	acetone	Yes	Yes	
Ul_MR_p05_H	MeRe	1:4	Yes	acetone	Yes	Yes	
Ul_phOH_p01_L	$Ph(OH)_3$	/	Yes	acetone	Yes	x	No CA
Ul_phOH_p02_L	$Ph(OH)_3$	1:1	Yes	$CDCl_3$	Yes	Yes	
Ul_phOH_p03_L	$Ph(OH)_3$	1:3	Yes	$CDCl_3$	Yes	Yes	
Ul_phOH_p04_L	$Ph(OH)_3$	1:6	Yes	$CDCl_3$	Yes	Yes	
Ul_phOH_p04_H	$Ph(OH)_3$	1:6	Yes	$CDCl_3$	x	x	
Ul_MROR_p02L	MC1	/	Yes	acetone	Yes	Yes	No CA
Ul_MROR_p02H	MC1	/	Yes	DMSO	x	x	No CA
Ul_MROR_p03L	MC1	1:1	Yes	acetone	x	x	
Ul_MROR_p03H	MC1	1:1	Yes	DMSO	x	x	
Ul_MROR_p05L	MC1	1:2	Yes	acetone	Yes	x	
Ul_MROR_p05H	MC1	1:2	Yes	DMSO	x	x	
Ul_MROR_p01L	MC1	1:2	No	acetone	x	x	Zulauf
Ul_MROR_p01H	MC1	1:2	Both	DMSO	x	x	Zulauf
Ul_MROR_p04L	MC1	1:4	Yes	acetone	Yes	Yes	
Ul_MROR_p04H	MC1	1:4	Yes	DMSO			
Ul_CA_p01L	none	0:1	Yes	$CDCl_3$	Yes	Yes	Pure CA
Ul_CA_p01H	none	0:1	Yes	DMSO	Yes	Yes	Pure CA

Table 3.4 resumes all the polymerization experiments, together with the performed analysis and other relevant information. The "_L" in the sample name indicates a light fraction, while a" _H" stands for a heavy one. The reported solvents are deuterated.

SEC data analysis

Figure 3.9 shows the SEC chromatogram of the CA DHP light fraction. The features of the chromatogram are not surprising, in fact the peaks at 28.7, 27.2 and 25.5 mL, are clearly visible. They are sign of the presence of coniferyl alcohol dimer, trimer and tetramer respectively. A small amount of free CA (**29**) is probably responsible for the peak at 29.8 mL. As SEC measures the hydrodynamic value of the molecules, the two shoulders 26.1 and 26.9 mL are probably caused by tetramers and trimers, respectively, with a different shape or somehow differently diffused through the column. Finally the big blob between 22 and 25 mL is caused by the high Mw (>1000 g/mol) molecules, which are out of the instrument's range. At that point the molecules can no longer be properly separated, but they still represent the weight distribution of the sample. As reported in the experimental chapter (p. 66 section 2.1.2), SEC calibration was attempted using polystyrene as standard. Unfortunately, not enough low Mw standards were available during the calibration, and the calibration curve ended up being imprecise. Therefore the precise value of retention volume is shown on the x axis of the chromatograms, instead of the unreliable Mw value. In order to give a rough evaluation of the involved Mw, we report the series of expected weights for CA polymerization. It is almost certain that the outcome of the performed reaction is CA polymerization and the data collected from SEC, even without a proper calibration, are consistent with this hypothesis. Therefore the proposed Mw values, which correspond to CA dimer, trimer and tetramers, should be reliable enough to function as a calibration. A coniferyl alcohol monomer weights 180 g/mol, when it couples with another monomer the weight increases depending on the formed bond. When a water molecule participates in the bond formation, as in a β-O-4 bonds (**34**), the weight increases of 196 g/mol. On the other hand, when two CA molecules react by themselves, the weight increases by 178 g/mol, as in a β-5 bond (**36**). Even if quantitative experiments haven't been performed, it appears from the NMR analysis that β-O-4 is not so abundant. Therefore the average of both values was used to calculate the Mw series, and the monomer addition was considered to be 187 g/mol. Of course, this is a simplification of the problem, but the values reported represent just a rough evaluation. The oligomer Mw series was calculated and it is reported in table 3.5, together with the assumed corresponding retention volume.

CA molecules number	1	2	3	4	5	6
Mw range (g/mol)	180	358-376	545-563	732-750	919-937	1106-1124
Retention volume (mL)	29.8	28.7	27.2	25.5	22-25	22-25

Table 3.5 reports the calculated Mw series for CA oligomers and their correlation with SEC peaks. The values reported are to be considered as an evaluation of the real Mw.

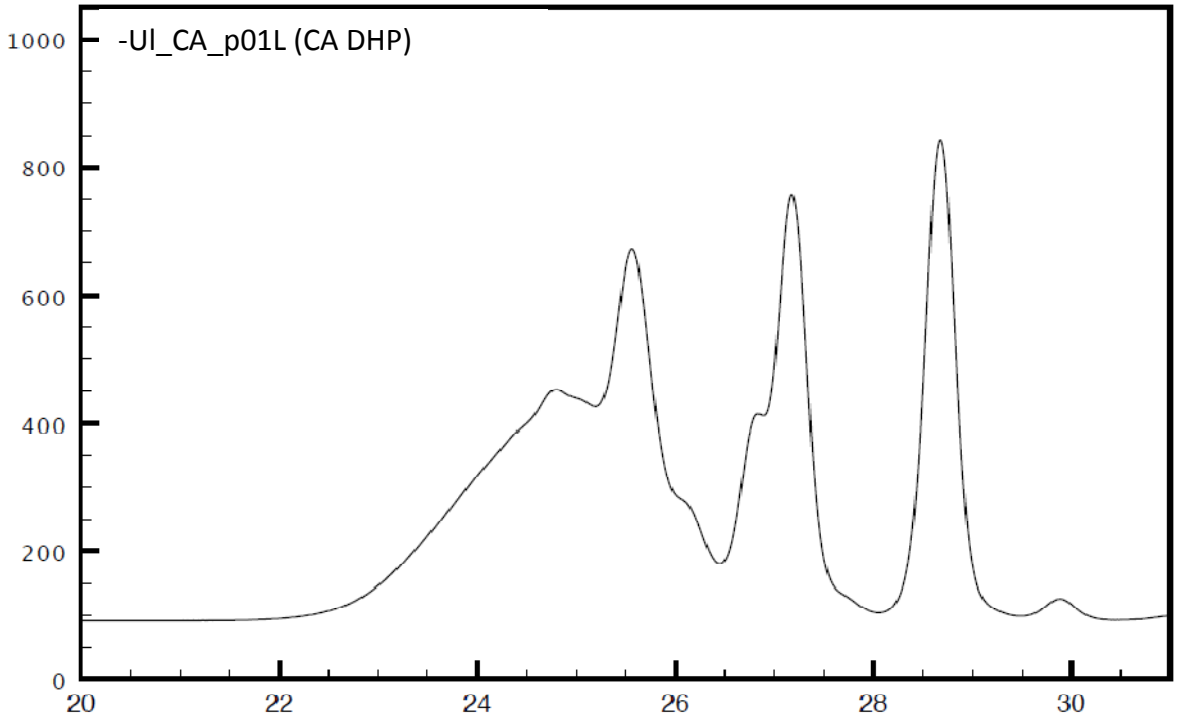
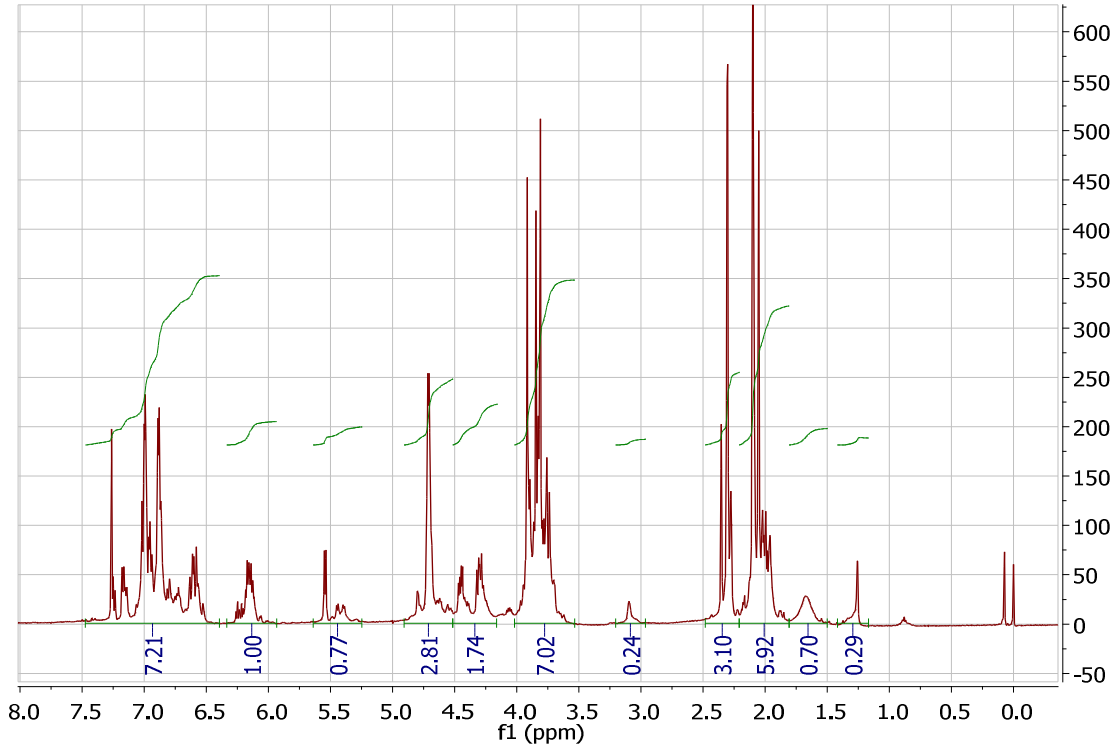


Figure 3.9 shows the SEC chromatogram of DHP light fraction. The results are the expected coniferyl alcohol dimers, trimers tetramers and higher oligomers.

NMR data analysis

Figure 3.10 shows the ^{1}H, ^{13}C and HSQC spectra of sample Ul_CA_p01L, the most useful spectra for peak assignment. Peak assignment was done not only by analysis of the recorded spectra, but also with the help of NMR databases [66,116,192] and our laboratory own knowledge. Just as expected from DHP, some of the most common structures in lignin were individuated, peak assignment is reported below, grouped by belonging structure. Due to the complexity of the spectra, the great amount of multiplets and amount of overlapping peaks, the multiplicity of the signals is not listed. A similar consideration was done for the aromatic region. As the aromatic protons do not play an active role in lignin links formation or reactions, the assignment of so many overlapping and unresolved peaks was considered a waste of time and energy.

a)



b)

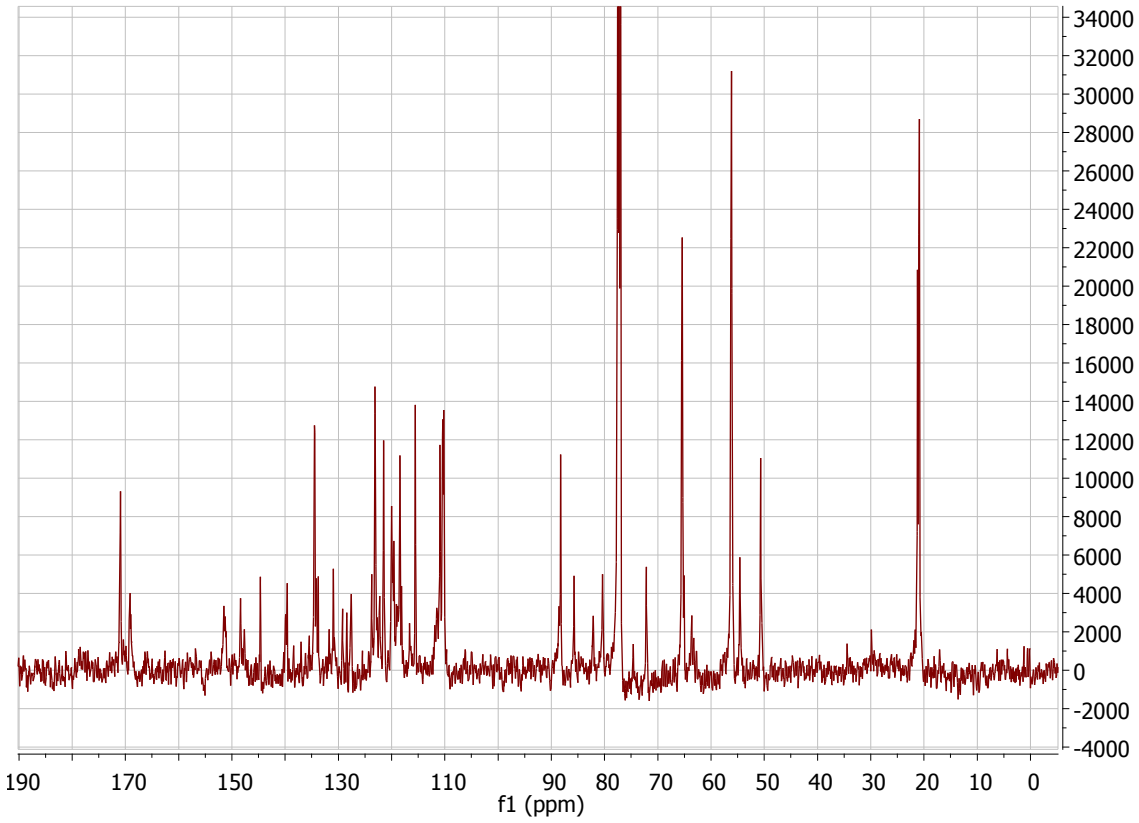


Figure 3.10 NMR spectra of CA DHP in $CDCl_3$+1%TMS: a) ^{1}H NMR b) ^{13}C NMR

c)

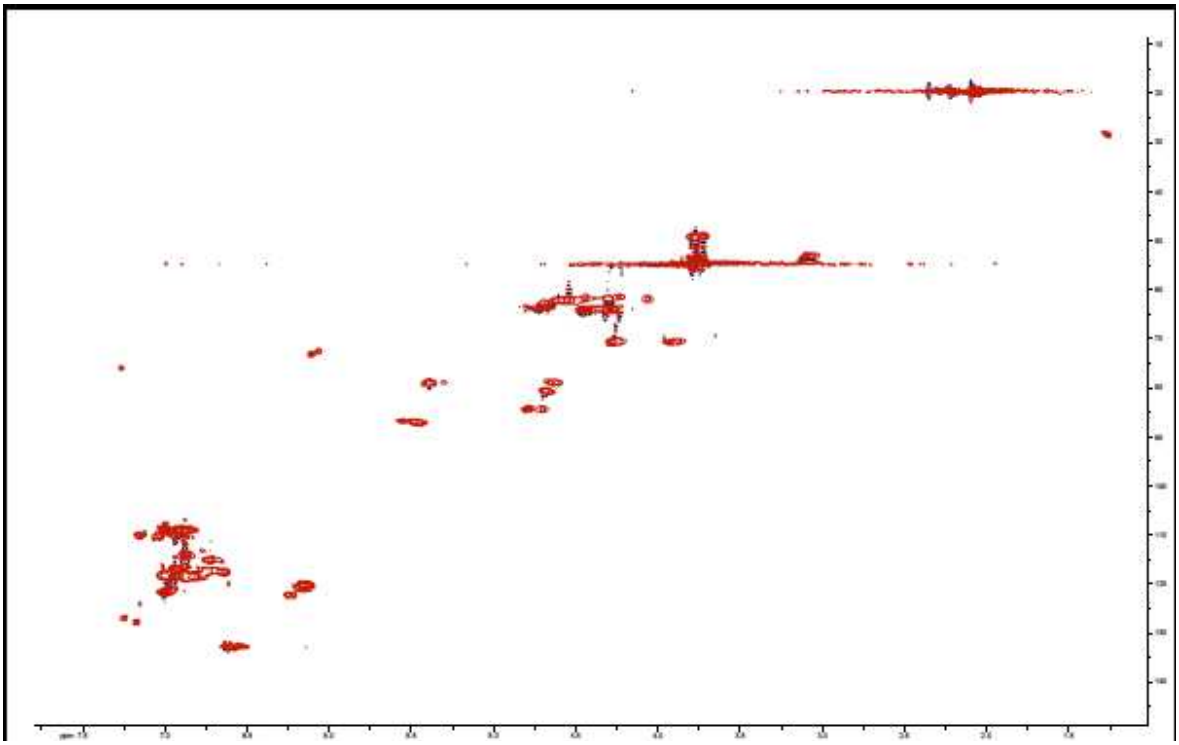


Figure 3.10 c) HSQC spectrum of CA DHP in $CDCl_3$+1%TMS:

α-O-4 β-5

- ^{1}H NMR : δ=5.5 ppm Hα, δ= 3.75 ppm Hβ, δ=4.28/4.45 ppm Hγ
-^{13}C NMR : δ=88.0 ppm Cα, δ= 50.47 PPM Cβ, δ= 65.15 ppm Cγ

β-β

- ^{1}H NMR : δ=4.70 ppm Hα, δ=3.09 ppm Hβ, δ=3.91/4.25 ppm Hγ
- ^{13}C NMR : δ=85.6 ppm Cα, δ=54.33 ppm Cβ, δ=71.85 ppm Cγ

β-O-4 α-O-4 trimer

-^{1}H NMR : δ=4.68 ppm Hβ, δ=4.54/4.62 ppm Hγ
- ^{13}C NMR : δ=82.0 ppm Cβ, δ=62.9 ppm Cγ

β-O-4 *threo*. Chirality was assigned based on previous study.

-^{1}H NMR : δ= 6.09 ppm Hα, δ= 4.63 ppm Hβ, δ= 4.04 (HSQC visible)/ ≈4.3 ppm Hγ
- ^{13}C NMR : δ=73.53 ppm Cα, δ=80.3 ppm Cβ, δ= 62.9 ppm Cγ

Free coniferyl alcohol propanoid end (unlinked/unreacted)

-^{1}H NMR : δ= 6.60 ppm Hα, δ= 6.15 ppm Hβ, δ=4.71 ppm Hγ

- ^{13}C NMR : δ= 133.7 Cα, δ= 121.22 Cβ, δ=65.32 ppm Cγ

Other relevant peaks

-metoxy groups: ^{1}Hδ= 3.6-3.9 ppm, ^{13}Cδ = 55.6

-methyl: ^{1}Hδ = 2.4-1.9 ppm, ^{13}Cδ= 20.9

-aromatics: ^{1} Hδ= 6.5-7.3 ppm, ^{13}Cδ= 109-150

-carbonyl: ^{13}Cδ= 170 ppm

-chloroform: ^{1}Hδ= 7.25 ppm, ^{13}Cδ= 77.0 ppm

This list includes most of the peaks present in the three proposed spectra. It is important to notice that the synthesized DHP is not very similar to lignin. Even if some of lignin's typical structures are present, many other are absent and the relative amount of each linkage is very different from in vivo lignin. Just to name a few examples, there is no trace of dibenzodioxocin rings (**38**) and β-O-4 (**34**) is present in low quantity. The obtained DHP is a satisfactory model for lignin for the current analysis.

Comparison strategy

Before moving on to the CM DHP data analysis, it is important to explain how the data was interpreted. Comparison of two SEC spectra is pretty straight forward, using one as a reference is it fairly easy to spot extra or missing peaks. Peaks shift was considered at first, but the Mw values obtained from calibration and the relation between Mw and retention volume are too unreliable too rely on this phenomenon. On the other hand, NMR was more complicated. In fact, due to the low solubility of the molecules, the solvent had to be changed for some of the samples. The different solvent causes some minor changes in the chemical shifts. Another problem is the great number of overlapping peaks. It is impossible to notice a difference between two spectra in the methyl or methoxy group region (^{1}Hδ = 2.4-1.9 ppm, ^{1}Hδ= 3.6-3.9 ppm). There are simply too many peaks, without a specific and fixed pattern, since the synthesis reaction is statistical. The same problem is present in the aromatic region, with the addiction of the central molecule. Whether the CM interacts with CA or not, it is still present in the reaction mixture and produces a signal, which needs to be taken into account. The CM signals are usually detectable, but in the aromatic region, where they overlap with all the CA (**29**) peaks, things can get

complicated. Mostly ^{13}C spectra were used for comparison, in order to overcome, or at least minimize, the resolution problem. The region between 60 and 100 ppm was analyzed with particular care. In this area are located all the carbons of CA propanoid chain, which are the most reactive and the ones most likely involved in bonds formation.

3.2.2 METHYLRESORCINOL

Table 3.6 summarizes the mass yields of light and heavy fractions obtained from the 5-methylresorcinol (**72**) experiments.

Sample name	CM/CA (mol/mol)	Yield (%)	Light fraction yield (%)	Heavy fraction yield (%)
Ul_MR_p01	1:0	130	130	0
Ul_MR_p02	1:1	63.93	50.44	13.49
Ul_MR_p03	1:2	73.89	63.57	10.32
Ul_MR_p06 (pH 4)	1:2	67.67	66.17	1.49
Ul_MR_p05	1:4	64.98	59.56	5.41

Table 3.6 resumes the yield obtained in the polymerization experiments using 5-methylresorcinol as a central molecule

The yield measured for sample Ul_MR_p01 is unreliable, most likely because not all the solvent was properly evaporated. This problem was encountered in every experiment without coniferyl alcohol, as can be seen in table 3.7 and 3.8 (pp. 113,117) from samples Ul_MROR_p02 and Ul_phOH_p01. The heavy fraction yield decreases with the CM/CA ratio. Changing the pH dramatically drops the yield of the solid fraction. Even without considering the heavy fraction data, MeRe experiments produced a high amount of spectra, too many to be individually discussed here. Therefore it was decided first to match each MeRe spectrum against the others, to see if they are all similar, and only after to compare one of them with the CA DHP analyzed above.

Methylresorcinol, internal comparison

Figure 3.11 shows the SEC runs of every MeRe (**72**) experiment. All runs appear to be similar, each chromatogram shows the same peaks without relevant shifts, even if with different ratios and intensities. As expected, these data suggests that the different reaction conditions did not strongly affect the outcome. The predictable result is that increasing the amount of CA (**29**) does not produce new bonds, but just increases the amount of the present structures.

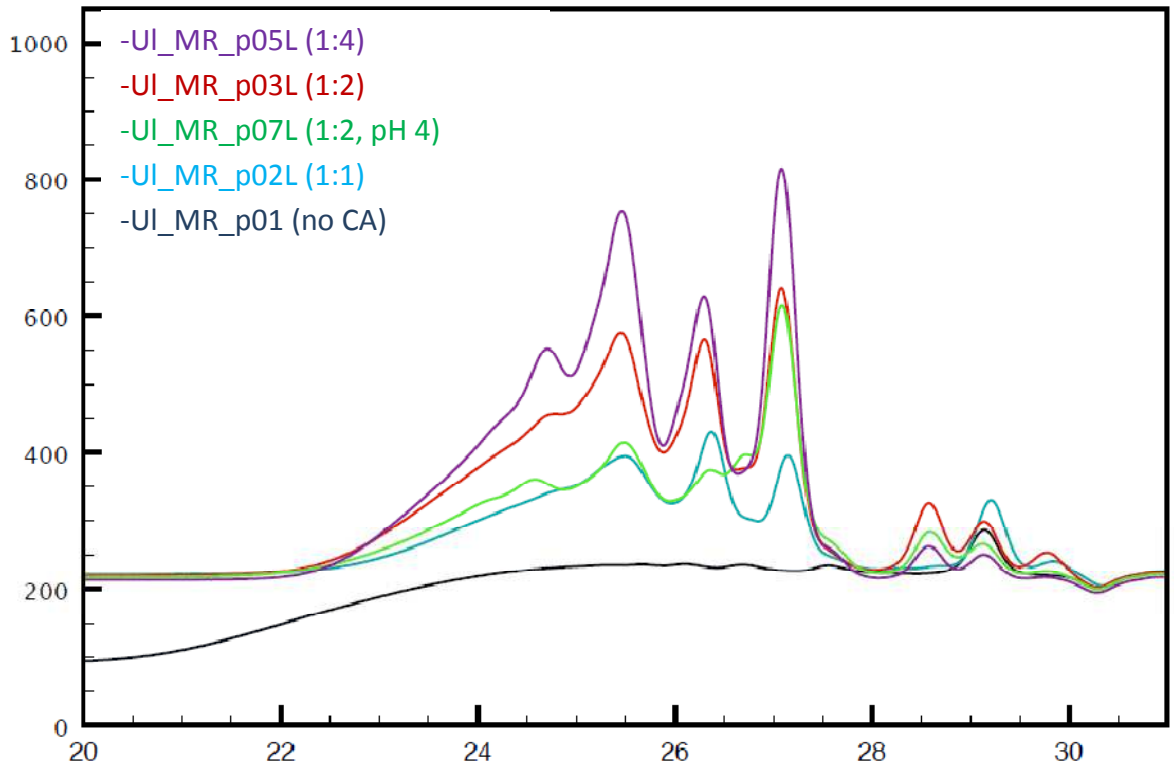


Figure 3.11 compares all the SEC runs obtained from the 5-methylresorcinol experiments. The only remarkable differences are in peak intensity, but all the peaks are always present and in the same position. This is a good indication that all the samples are very similar.

This conclusion is supported by NMR evidence as well. Figure 3.12 shows the ^{13}C spectra of the MeRe light fractions. The spectra display mostly the same peaks in every experiment, with few minor differences. Some peaks can be seen only in the experiments with high amount of CA. This is probably just an effect of concentration and not the evidence of new bonds formation. With a too low amount of CA some of the structure are not enough concentrated in the sample to be detected by the very insensitive ^{13}C experiment. In any case sample Ul_MR_p03_L (ratio 1:2) was used for comparison with Ul_CA_p01L. Even if it is not the sample with the highest CA concentration, Ul_MR_p03_L was chosen because it clearly shows every relevant peak and displays a higher resolution than the other samples in most of the NMR experiments.

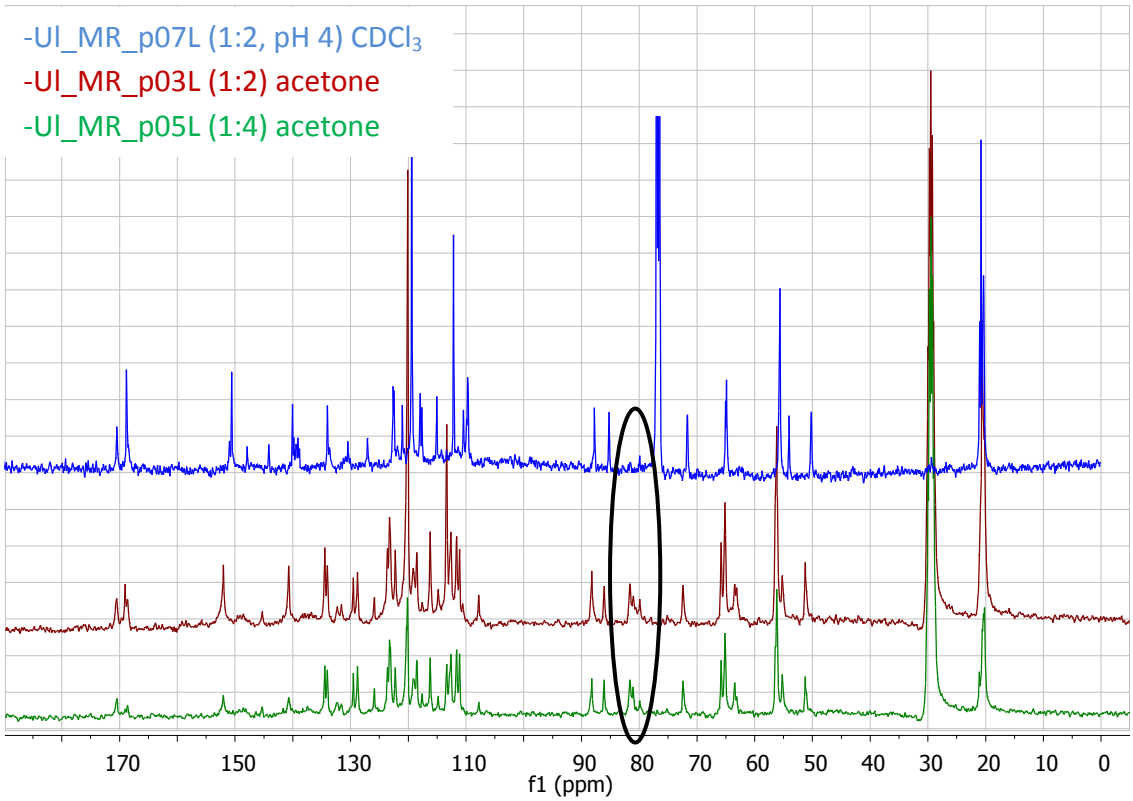


Figure 3.12 compares the different DHP obtained from MeRe experiments. The samples with low CA content (1:0, 1:1) show few signs of polymerization and have been excluded. It is possible to observe that the 3 spectra are very similar. Sample Ul_MR_p07L is slightly different from the other two (80 ppm), both because different reaction conditions and different solvent. The other two spectra appear to be almost identical.

Methylresorcinol, CA DHP comparison

Ul_MR_p03_L, representative of every MeRe (**72**) experiment, and Ul_CA_p01L, our DHP reference, are compared here. Figure 3.13 shows the SEC chromatograms of the two samples. The two spectra appear to be quite similar, as expected, but with some relevant differences: a peak at 29.2 mL and another at 26.3 mL. It is also remarkable the difference in intensity of the peak at 28.7. The first peak at 29.2 mL is most likely caused by the unreacted CM, as shown by the CA free sample Ul_MR_p01_L. On the other hand, the peak at 26.3 mL is probably caused by a CM-CA compound. The decrease in intensity of the CA dimer peak (29.2 mL) could also point in the direction of a reaction involving CA.

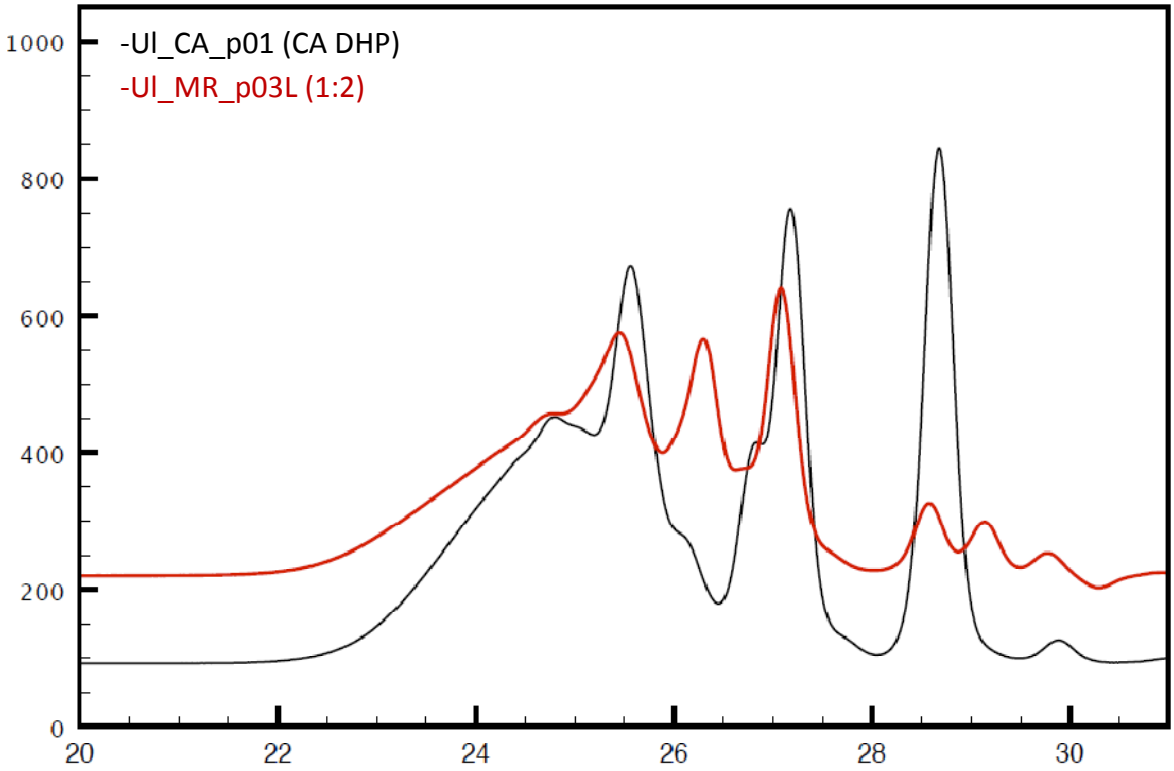
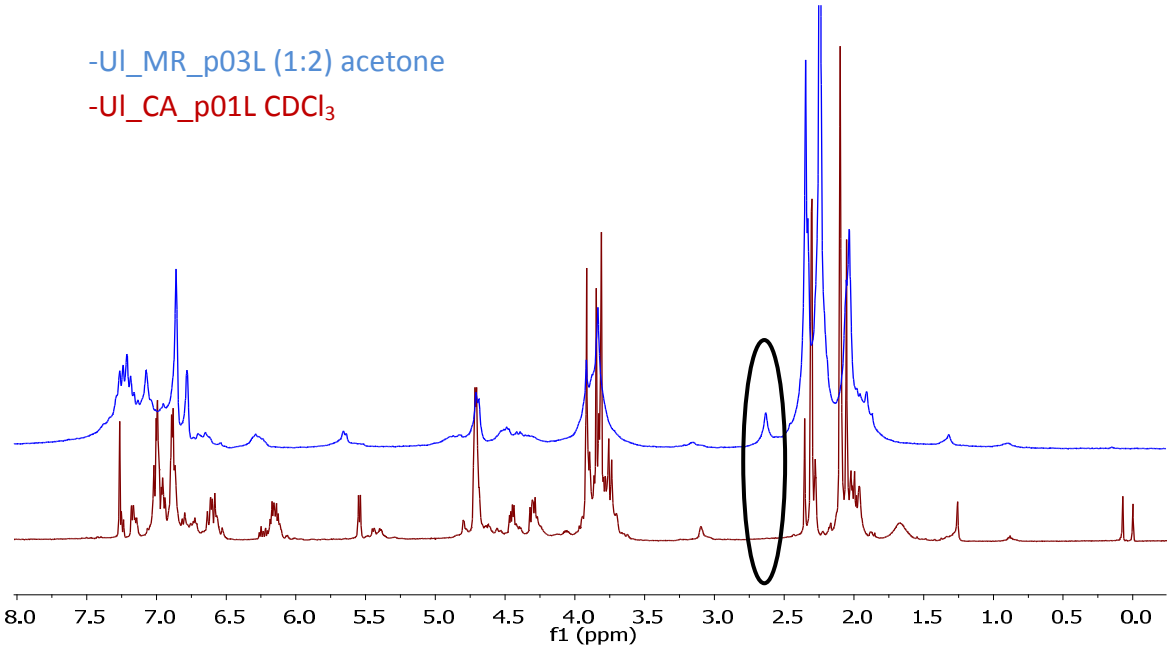


Figure 3.13 compares the SEC chromatogram of regular DHP and MeRe DHP. There are some relevant differences, first of all an extra peak at 26.2 mL, which could be the sign of CM-CA coupling.

Figure 3.14 compares the relevant NMR spectra, in particular ^{1}H, ^{13}C, and HSQC. Even if the spectra look fairly different there are two important considerations to be done. The first is the difference in solvent. Ul_CA_p01L is recorded in $CDCl_3$, while Ul_MR_p03_L was dissolved in acetone (d_6). It is therefore necessary to ignore the solvent peaks when comparing the two spectra, and take into account a little chemical shift difference for some peaks. The second big difference, resolution, is caused by the equipment employed to record the spectra, 500 MHz for the CA DHP and 300 MHz for the MeRe DHP. Most of the differences in peak shape are just an effect of low resolution.

Comparison of the two ^{1}H spectra reveals few differences if the peak positions are considered instead of peak shapes, and the few visible extra peaks do not belong to CM-CA product. The differences in the methyl and aromatic region (2 and 7 ppm) are caused by the different solvents and by the central molecule. The two small peaks at 1 ppm, present in both samples, belong to some non influent impurity. Finally, the extra peak at 2.6 ppm might appear promising, but it is just another acetylated alcohol, as suggested by the HSQC spectrum. The peak positions between 3 and 6.5 ppm appear well matched, but even if an extra peak was present it would be impossible to notice it because of the low resolution. The overall analysis of the ^{1}H spectrum is inconclusive, as expected. In the ^{13}C spectra four remarkable differences are found, three of them in the aromatic region. Compared to the CA DHP, sample Ul_MR_p03_L displays two extra peaks at 120 and 108 ppm, together with a difference in the peak at 113 ppm. The extra peaks at 120 and 113 are explainable by the presence of 5-methylresorcinol, as can be seen in Figure 3.14. On the other hand, the origin of the peak at 108 ppm is unknown. The resolution of the HSQC spectrum is too low to enable investigation of the overcrowded aromatic area. Finally, the last difference between the two spectra is a small extra peak at 65.8 ppm. This is exactly the region where evidence of a CA-CM link was expected. Unluckily, the origins of this peak cannot be further investigated, because of the HSQC spectrum bad resolution. In fact many peaks of γ carbons overlap in that area, and it is hard to tell which proton signal correlates with our extra peak. Few others minor differences are present in the spectra (small ppm shifts or peak shaper difference), but the HSQC spectrum does not show anything out of place in the corresponding positions.

a)



b)

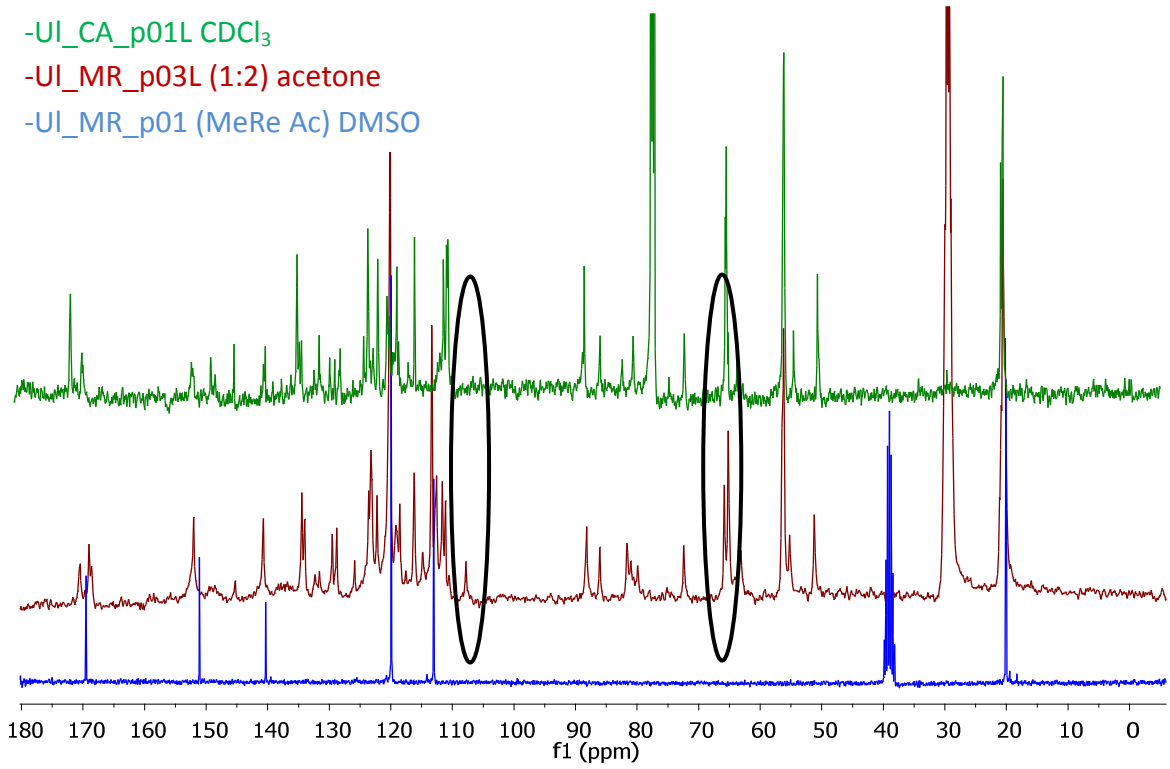


Figure 3.14 shows the spectral comparison between MeRe DHP and CA DHP. The main differences are circled in black and suggest that some CM-CA interactions are taking place. a) ^{1}H NMR b) ^{13}C NMR

3.2.3 PHLOROGLUCINOL

Table 3.7 summarizes the mass yields of light and heavy fractions obtained from the phloroglucinol (**76**) experiments.

Sample name	CM/CA (mol/mol)	Yield (%)	Light fraction yield (%)	Heavy fraction yield (%)
Ul_phOH_p01	1:0	105.6	105.6	0
Ul_phOH_p02	1:1	52.99	52.99	0
Ul_phOH_p03	1:3	89.32	89.32	traces
Ul_phOH_p04	1:6	72.60	67.39	5.20

Table 3.7 resumes the yield obtained in the polymerization experiments using phloroglucinol as a central molecule

The yield of the first experiment is unreliable. The heavy fraction yield shows an opposite pattern compared to MeRe (**72**) and the overall yield appears to be quite random. Just as in the previous section, first every $Ph(OH)_3$ spectrum will be compared with the others, then the best one will be chosen and matched against the blank DHP.

Phloroglucinol, internal comparison

Figure 3.15 and Figure 3.16 show the SEC and NMR runs performed on phloroglucinol (**76**) samples. The similarity between these samples can easily be seen, the only exception is one extra peak at 30 ppm in the NMR spectrum, in sample Ul_PhOH_p02. This peak correlates in the HSQC spectrum with the methyl protons at 2 ppm. Despite the unusual position, this peak is most likely originated by acetylation, and therefore not relevant. Sample Ul_phOH_p04_L was used for comparison with Ul_CA_p01L, for the same reasons discussed above. An important feature of the SEC chromatograms is the presence of a peak at 26.1 mL in every sample.

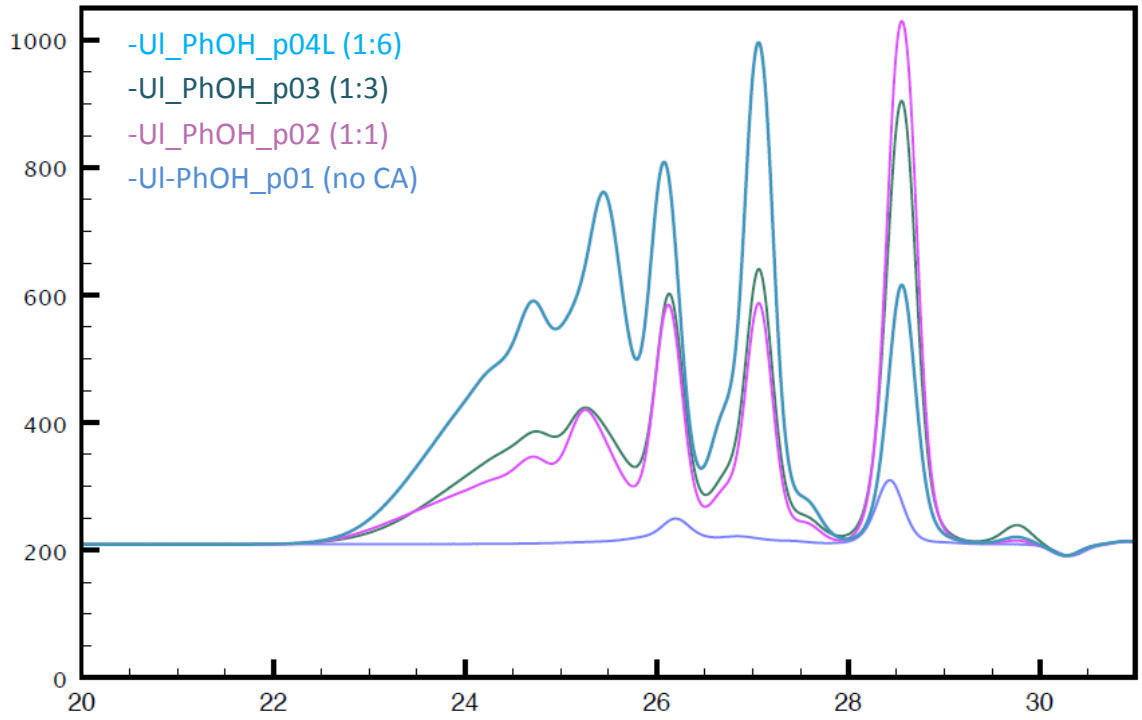


Figure 3.15 compares all the SEC runs obtained from the phloroglucinol experiments. The only remarkable differences are in peak intensity.

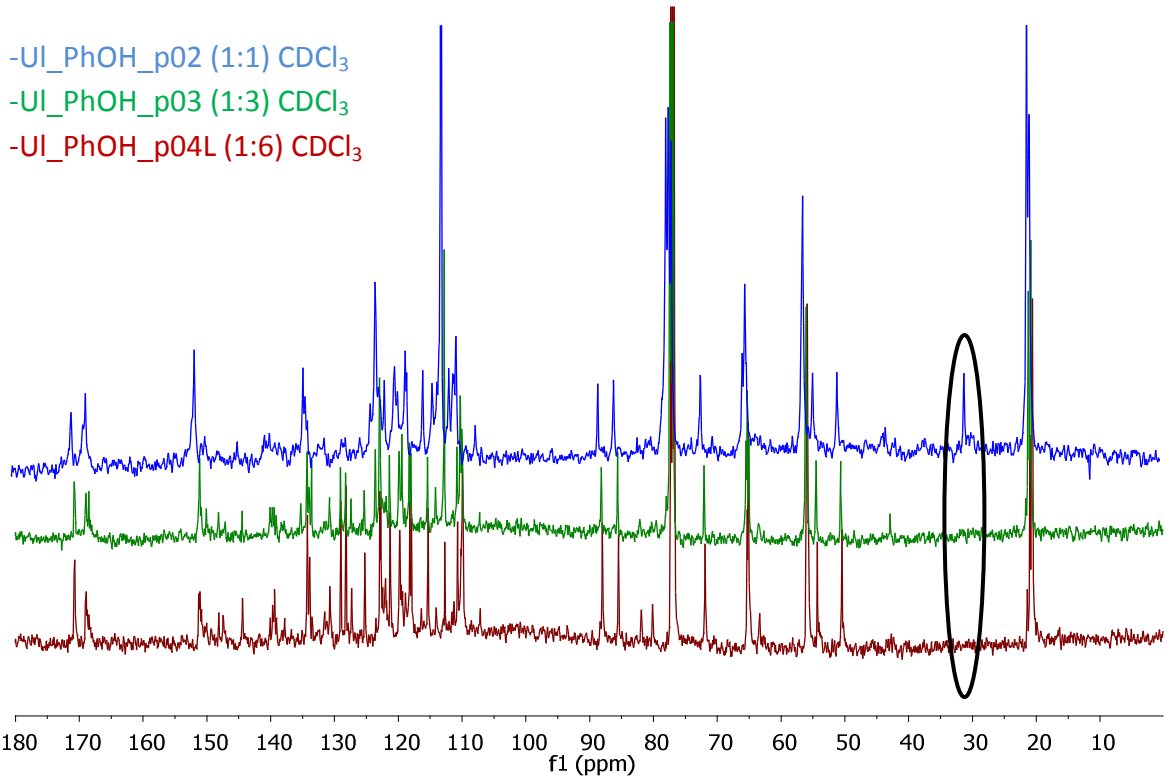


Figure 3.16 Compares the phloroglucinol DHP ^{13}C NMR spectra. The only difference is circled in black, but analysis of the HSQC spectra reveals that the peak at 30 ppm is probably caused by acetylation.

Phloroglucinol, CA DHP comparison

Figure 3.17 compares the SEC chromatogram of samples Ul_phOH_p04_L and Ul_CA_p01L, the only noticeable difference is the peak at 26.1 mL and its origin is uncertain. On one side, the presence of the same peak in the CA free experiment is a hint that this peak should not be considered. On the other hand, the peak intensity increases with the amount of CA (**29**), which is possibly a proof of CA involvement. Also the position of the peak (26.1 mL, similar to the assumed CA-MeRe cross coupling product) points in the same direction. Finally, a retention volume of 26.1 mL is too low for a simple acetylated $Ph(OH)_3$, the peak might belong to a dimer or formed by other side reactions, but there is no evidence of it in the NMR.

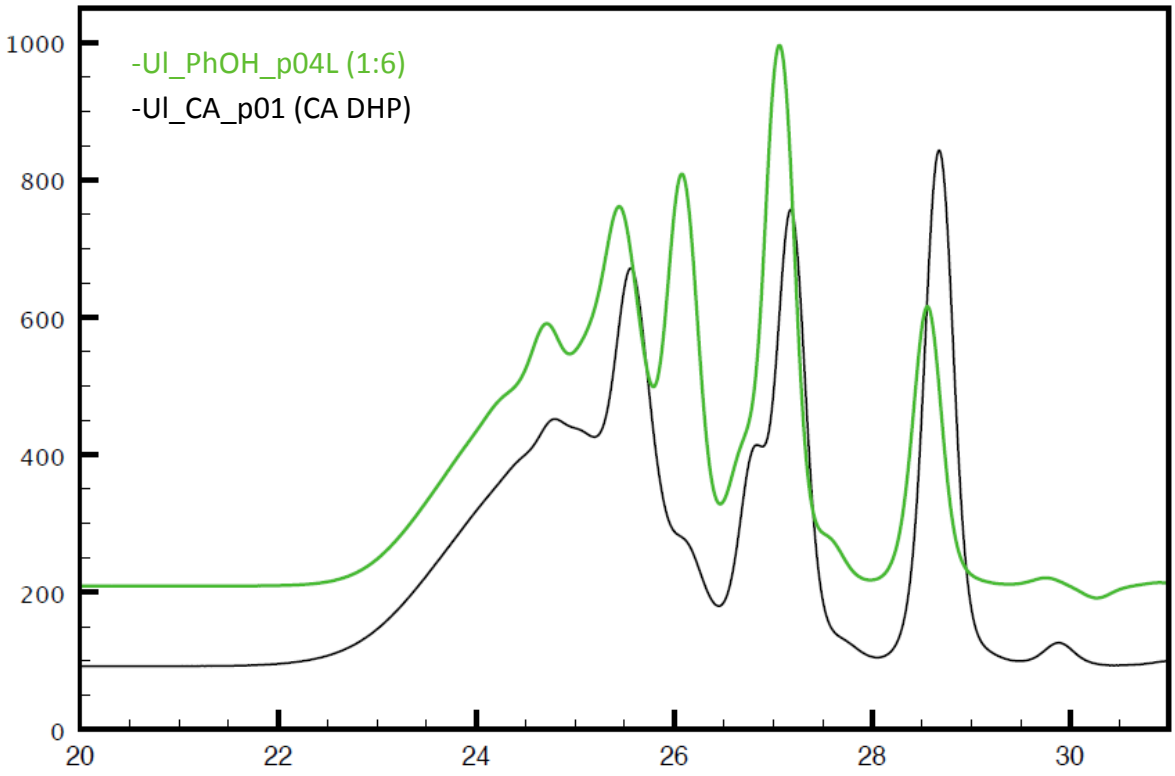
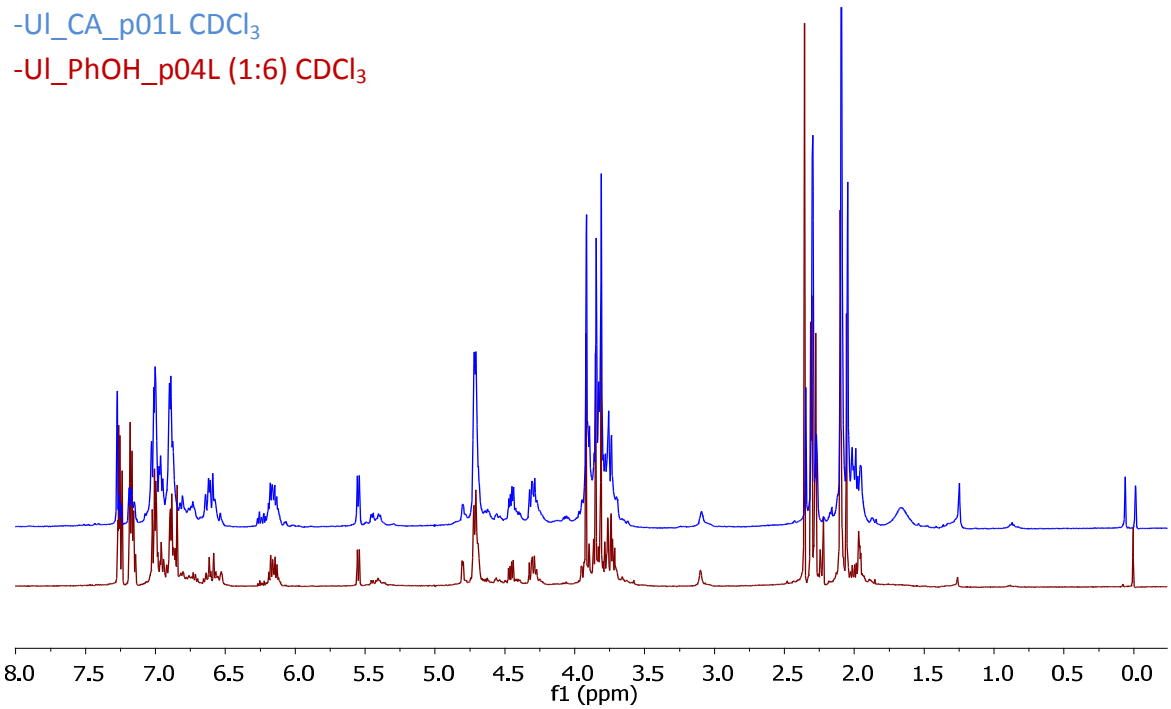


Figure 3.17 compares the SEC chromatogram of regular DHP and $Ph(OH)_3$ DHP. The origin of the peak at 26.1 mL is uncertain.

As the SEC data cannot provide a clear answer, it is important to analyze the relevant NMR spectra, shown in Figure 3.18.

a)



b)

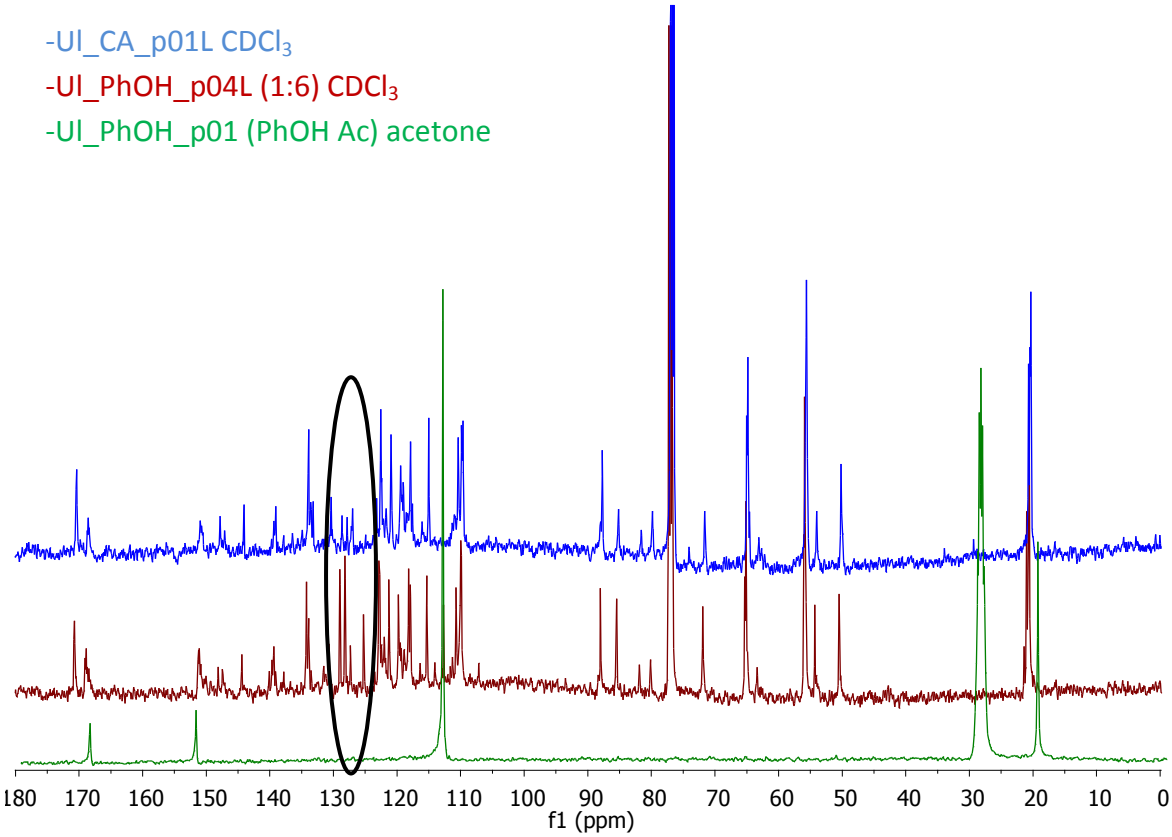


Figure 3.18 evidences some differences between $Ph(OH)_3$ DHP and CA DHP, all of them in the aromatic area (7.2 ppm, 125-128 ppm). The resolution is too low for further investigation. a) ^{1}H NMR b) ^{13}C NMR

In this case the comparison is easier because both samples were dissolved in $CDCl_3$ and processed with a 500MHz NMR machine. For what concerns the ^{1}H spectra, the only dissimilarity between the two is a difference in intensity of the peak at 7.17 ppm, while the ^{13}C spectra evidences a two extra peaks at 112.7 and 125.27 in the $Ph(OH)_3$ samples, and a significant intensity difference in the peaks around 128 ppm. The peak at 112.7 belogns to the unreacted $Ph(OH)_3$, as can be seen from Ul_phOH_p01_L ^{13}C spectrum, shown in Figure 3.18. The other peaks are aromatic carbons, probably linked to protons at 7.18-7.25 ppm, according to the HSQC spectrum. The high amount of peaks crowded in the aromatic area makes it complicated to assign those peaks with certainty. The ^{13}C spectra of Ul_phOH_p04_L and Ul_CA_p01L are almost identical between 20 and 110 ppm, i.e. anywhere but in the aromatic region.

It is hard to tell whether a CM-CA cross coupling is actually happening. On one hand both techniques show promising signs (one extra peak and peak shift in the SEC chromatograms, differences in NMR spectra). On the other hand, there is always something that makes questionable the validity of these data (Ul_phOH_p01_L SEC run, lack of NMR evidence in the propanoid chain region).

3.2.4 MODEL COMPOUND 1 (MC1)

Table 3.8 summarizes the mass yields of light and heavy fractions obtained from the MC1 (**75**) experiments.

Sample name	CM/CA (mol/mol)	Yield (%)	Light fraction yield (%)	Heavy fraction yield (%)
Ul_MROR_p02L	1:0	107.16	70.0	37.16
Ul_MROR_p03L	1:1	67.98	15.17	52.80
Ul_MROR_p05L	1:2	83.14	34.35	48.78
Ul_MROR_p04L	1:4	54.58	13.50	41.08

Table 3.8 resumes the yield obtained in the polymerization experiments using MC1 as a central molecule

The yield measured in the first experiment is unreliable. What is surprising here is the unexpectedly high amount of solid fraction produced by these experiments. Another interesting feature is the reaction of sample Ul_MROR_p02, the CA free reference. In fact, simple reaction of MC1 with H_2O_2 and HRP, produced an heavy fraction. Most likely MC1 is coupling with itself and producing some sort of polymer, while only the unreacted acetylated MC1 and possibly some dimers remain in the liquid fraction. To have a more reliable reference, MC1 was simply acetylated, without reaction with H_2O_2.

MC1, internal comparison

The few SEC chromatograms available are reported in Figure 3.19 while Figure 3.20 shows the NMR spectra. The only difference is present in the NMR spectra. Sample Ul_MROR_p05 appears to be slightly different from the others, in fact some peak appear to be much more intense in this sample. This phenomenon is caused by the higher concentration of unreacted acetylated MROR. As the other features are similar for every spectrum, sample Ul_MROR_p04 was chosen for comparison with CA DHP.

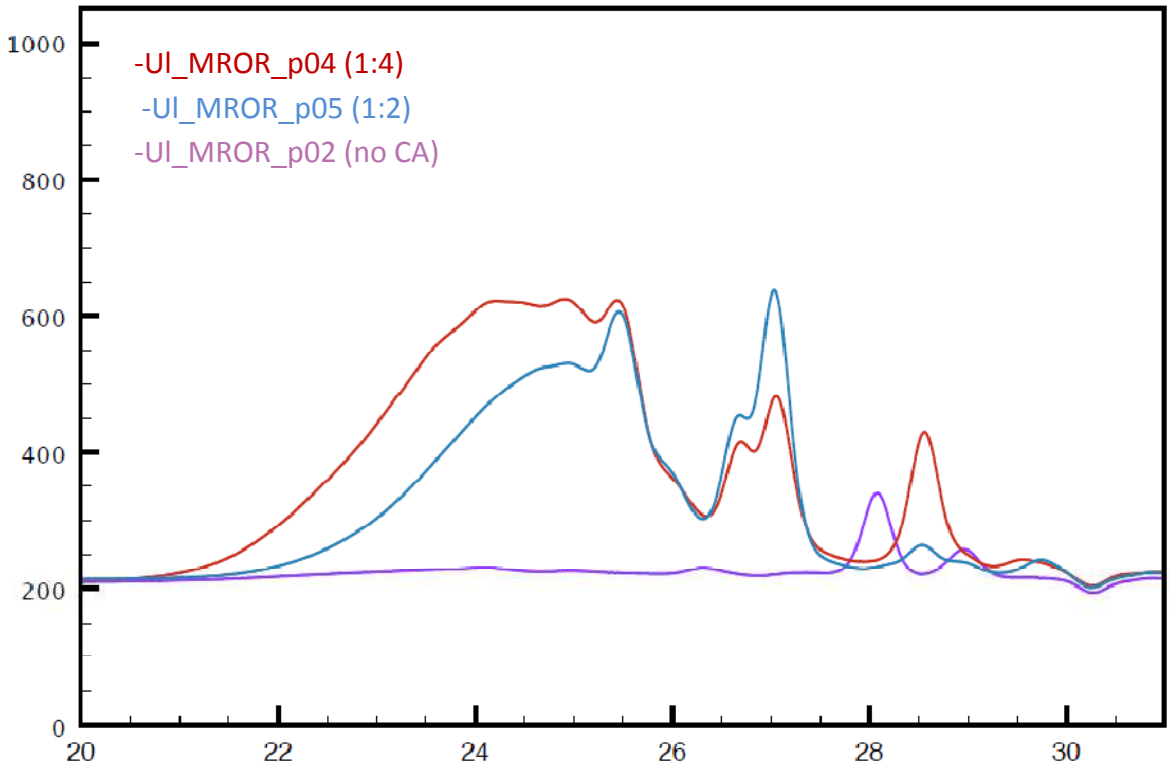


Figure 3.19 compares all the SEC runs obtained from the MC1 experiments. Reaction of MC1 with H_2O_2 yields some unknown product, shown at 28 mL. the other samples appear similar to each other.

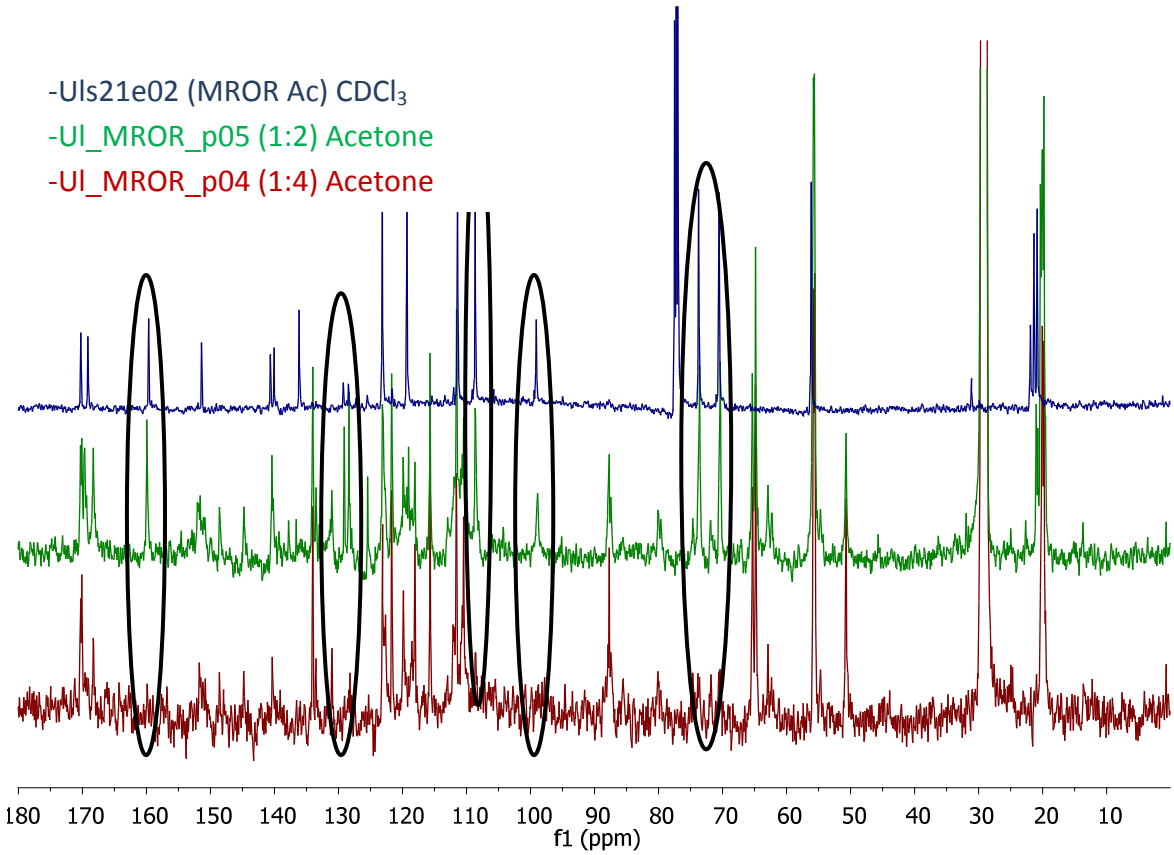


Figure 3.20 Comparison of the ^{13}C spectra clearly shows that the differences between samples Ul_MROR_p05 and Ul_MROR_p04 are caused by unlinked MC1. All the other features are extremely similar.

MC1, CA DHP comparison

This is probably the easiest of the analysis, and the most disappointing. Figure 3.21 compares the SEC experiments, and it can be easily seen how similar the MC1 samples are to the CA DHP. A similar conclusion can be drawn by looking at Figure 3.22 which shows the relevant NMR spectra.

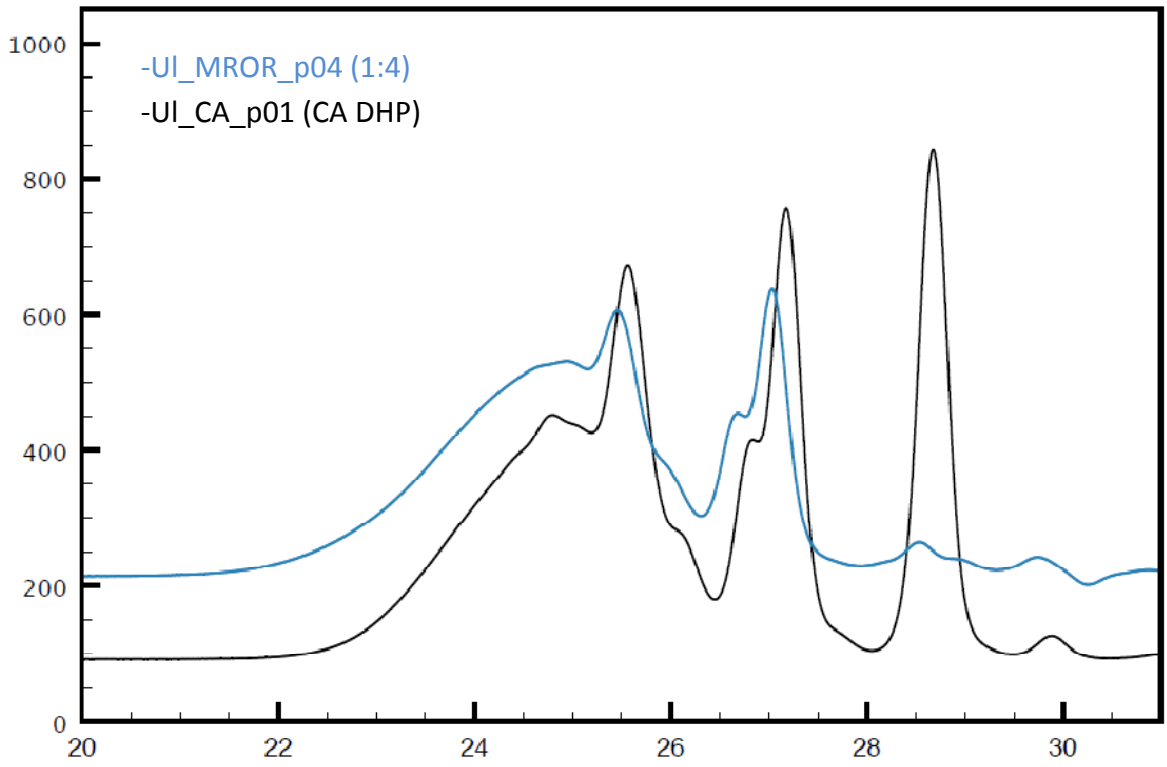


Figure 3.21 compares the SEC chromatogram of regular DHP and MC1 DHP.

a)

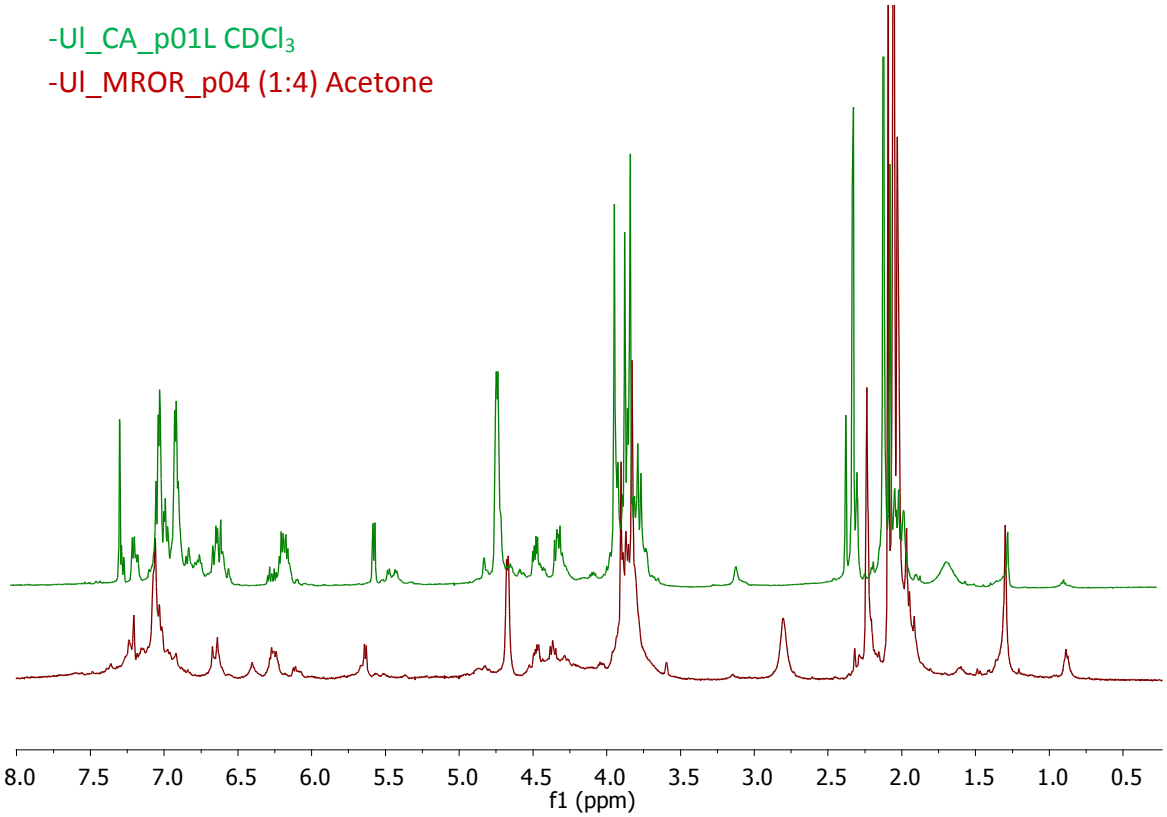


Figure 3.22 a) ^{1}H NMR spectral comparison of CA DHP and CM1 DHP.

b)

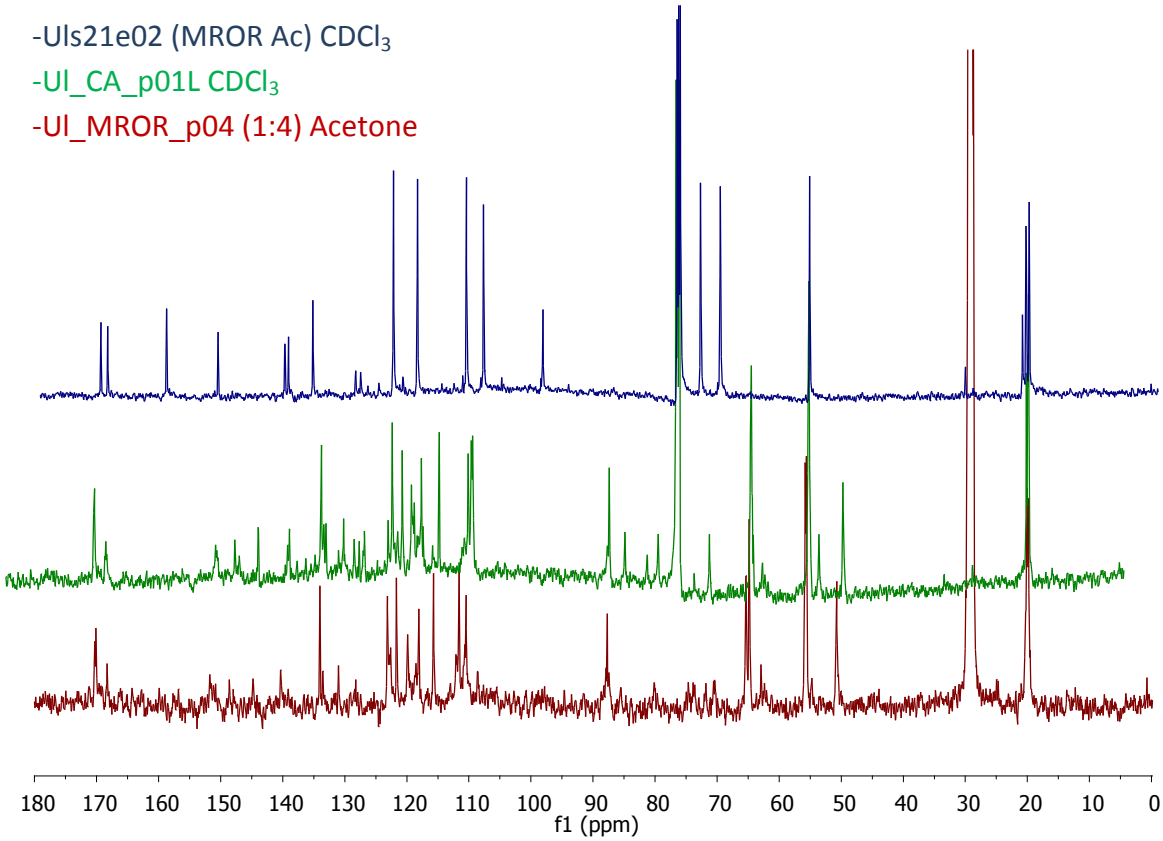


Figure 3.22 b) ^{13}C NMR spectral comparison of CA DHP and CM1 DHP. Some differences are present, but the MC1 DHP always displays less peaks that the reference DHP.

Comparison between the NMR spectra of samples Ul_CA_p01L and Ul_MROR_p04L is made harder by the different solvents and NMR equipment employed, which slightly shifts some of the peaks and changes the resolution. When comparing both the ^{1}H and the ^{13}C spectra, it is possible to notice several differences between Ul_MROR_p04L and Ul_CA_p01L. Unluckily, in every case, the CA DHP shows more peaks that the CM DHP sample. This is probably due to concentration effects. Sample Ul_MROR_p04L is much less concentrated, which means that many peaks in the ^{13}C spectrum are either undetectable or absent. In particular CM DHP shows fewer peaks and lower peak intensity in the area between 65 and 84 ppm. The peaks at 64.8 and 55.6 appear to be shaped differently from the reference sample, but there is no evidence of anything out of place in the HSQC spectrum. The aromatic areas are similar in the ^{13}C spectra, but some differences in intensity are visible in the ^{1}H. The only interesting difference between the two samples is an extra peak at 2.8 ppm in the ^{1}H spectra. Unfortunately this peak is most

likely caused by water impurity, as it doesn't correlate to any carbon in the HSQC spectrum. The NMR and SEC data combined suggest that no cross coupling is happening in the light fraction of these experiments. Due to the high yield of the heavy fraction it is possible that some more interesting features could be discovered there, but a more complete understanding of the reaction and of the NMR spectra is needed before it will be possible to properly investigate it.

3.2.5 CONCLUDING REMARKS

To analyze the huge amount of data produced by the polymerization experiments, it was decided to proceed by comparison. A pure coniferyl alcohol DHP was synthesized, using the same reaction condition as every other polymerization. Then it was thoroughly analyzed and most of the peaks were assigned. Finally, this CA DHP was used as a reference to analyze the DHP synthesized in presence of a central molecule. This way it was possible to simply look for differences instead of reassigning every peak each time. Different results were obtained with the three central molecules. Experiments with MeRe (**72**) were pretty satisfactory, both SEC and NMR evidence support the presence of a CM-CA product. Unfortunately the resolution of our analysis was not enough to get a definitive proof. The situation is different for the DHP obtained with phloroglucinol (**76**). The data suggest that something is happening, an extra peak is definitely present in the SEC run and some relevant differences are visible in the NMR spectra. Because of some contradiction in the obtained data, it is uncertain whether these features are produced by CM-CA cross coupling or by some other side reaction. Finally, MC1 (**75**) was the central molecule on which rested the highest expectations and turned out to be the most disappointing. Apparently both SEC and NMR indicate that our CM and CA are not interacting. The only hope is the high amount of heavy fraction produced, a different behavior compared with the other experiments. It is possible that cross coupling between CM3 (the heavier of the CMs) and CA produces an insoluble molecule, but for now it is only speculation. Only the light fractions were examined during this study, because of the technical problems connected with analyzing the heavy insoluble part of DHP. As the study of these molecules is just beginning, we decided to focus on something that could be handled and treated properly, instead of going in over our heads with unknown and hardly analyzable samples. Only with a proper purification strategy or a specific analysis method it will be possible to accomplish something in the study of the heavy fractions, but it will be possible to do it

only once the light fractions are better understood and with the help of a more complete NMR database.

Finally it is necessary to discuss the polymerization yields. In the previous paragraphs only a little time was devoted to this topic, because a valid hypothesis which could explain their behavior was not found. The overall yield appears to behave very randomly, with some relation with the central molecule or the amount of CA (**29**). The light or heavy fractions show some signs of a pattern, but really vague and hardly identifiable. They appear to be definitely connected to the central molecule, but it is hard to tell why or how, and they do not seem to be dependent on anything else. The most logical explanation for this phenomenon is a poor experimental procedure. The small amount of matter involved, the low solubility and stickiness of the heavy fraction made the complete recovery of the products complicate, and therefore the yield data less reliable. This is probably the main reason behind the random yield values obtained and therefore the data was used with caution.

4 Conclusions

It is hard to write a proper conclusion for this thesis, because many things changed from the beginning to the end of the work. Objectives and expectations, results and goals have been reconsidered along the way. The conclusion usually answers to the questions posed in the beginning, but it is impossible to do it here. Every initial question is still without an answer, this research only produced some preliminary results.

4.1 Summary

Every result has already been presented, resumed and discussed. They will just be summarized in here once more.

The aim of the work was to synthesize three new model compounds for lignin, MC1 (**75**), MC2 (**78**) and MC3 (**71**). Once synthesized, they were to be used as central molecules to start coniferyl alcohol (**29**) polymerization, to produce DHP. The aim was to investigate how the presence of these CMs would influence DHP growth, specifically if it would generate a closed ring, formed by the junction of two polymer growing chain. Identification and characterization of the closed loops could help understanding the growth mechanism of natural lignin.

4.1.1 SYNTHESIS

The synthesis of the proposed model compounds yielded different degrees of success:

1. MC1 (**75**) was successfully synthesized with an overall yield of 35%. The performances of the reaction could be greatly improved by optimizing the recrystallization purification step.
2. MC2 (**78**) was synthesized, purified and characterized, but with a yield too low to be employed in further experiments. Because of data misinterpretation most of the optimization work conducted on this reaction was useless. Therefore, some work performed in the right direction would most likely improve this reaction's performances.

3. MC3 (**71**) was never synthesized. Probably because of steric hindrance, the condensation step of the reaction never worked properly and the reaction was abandoned. Some alternative reaction routes have been proposed.

More details and a deeper discussion are found in the appropriate sections.

4.1.2 POLYMERIZATION

Because of the troubles encountered in the synthesis of MC2 (**78**) and MC3 (**71**), the initial plans had to be changed. It was decided to use MeRe (**72**), $Ph(OH)_3$ (**76**) and MC1 (**75**) as central molecules for DHP polymerization instead. Most of the experiments produced both a soluble and insoluble fraction of DHP (light and heavy). Because of the technical difficulties connected to the heavy fraction, only the light fraction was considered and analyzed in this study. Due to the complexity of the collected data, the purpose of the work became looking for signs of CM-CA cross coupling more than evidence of "closed loops". The results obtained by the polymerizations depend on the employed central molecule:

1. MeRe DHP (CM1 DHP) showed encouraging results. Both NMR and SEC data support the existence of CM1-CA cross coupling, but the low resolution of the NMR spectra does not allow further investigation.
2. $Ph(OH)_3$ DHP (CM2 DHP) shows similar signs, but the data is a bit more controversial. Some reaction other than CA coupling is surely happening during the polymerization, it is hard to tell whether it is CM2-CA cross coupling or some other side reaction.
3. MC1 DHP (CM3 DHP) is the least interesting. No signs of cross coupling appear in the light fraction, but the high yield of the heavy fraction might be a hint that some reaction is taking place there.

4.2 A Preliminary Work

Since the beginning of the project it was not clear what kind of signal the closed loops would produce, or how to detect and analyze them. There was so much work to be done before the polymerization experiments could even be started, that detection of the closed loops was not a great concern. It was uncertain whether a viable model compound could be synthesized, the initial plan was just to try some polymerization experiments and see what happened. In the end, detection of the closed loops was an objective too ambitious for the time at our disposal, and the aims of the project stopped at the cross coupling step. It is important to be sure that the model compound and CA interact together, before analyzing what the interaction produces. Moreover, the detection of cross coupling and the identification of the related peaks is the first step to detect the more complex structures of the closed loops.

It is possible that our project was too ambitious in the first place, that even in the best condition it would require more time than available, but the lack of the expected results was also caused by the unexpected problems that were faced. It is possible to divide the obstacles encountered in this project between expected and unexpected. The lack of results in MC3 condensation step was somehow expected. The high steric hindrance present in the condensation step was an obvious obstacle from the start, even if it was unknown whether it could be overcome or not. Another totally foreseeable difficulty was the low resolution of the NMR spectra. Even without knowing what kind of signal the closed loops would produce, it was predictable that the great number of peaks and the high Mw of the molecules would complicate the work. On the other hand, the probably biggest problem of the whole project was almost completely unforeseen. Because of the size of the molecules involved, it was expected that solubility could cause some troubles. In hindsight, it is obvious that the problem was deeply underestimated. Solubility, instead of being a minor issue, ended up being a constant trouble. During the synthesis, each step required more time than expected because the right solvent had to be found, and some of the molecules were never completely soluble. This forced us to go to longer reaction times and harsher condition than usually employed. Another synthesis problem was linked to the use of TLC, it’s hard to properly follow a reaction when half of the reaction mixture is not soluble in any eluent. The same problem is relevant for separation techniques, like chromatographic columns, extractions and sometimes even recrystallization. Our work would have been

much easier if the heavy DHP fraction could have been dissolved and separated by HPLC. The low solubility heavily influences the samples analysis as well. Probably the whole MC2 misinterpretation (p. 88) wouldn't have happened with a completely soluble sample, and ^{13}C NMR analysis would have been much faster and useful with higher amount of product dissolved in the sample. Solving or finding a way around the solubility problem will be a key step for the further development of this research.

4.3 Future Work

Most of the proposals for the future work will be a simple repetition of our initial aims. This study has to be considered as a preliminary work, the gathering of the first data necessary to achieve the initial goals. The search of the same results is just the natural continuation of this project.

4.3.1 SYNTHESIS

The future prospects for the synthetic work have already been discussed in the results and discussion chapter. For what concerns the proposed molecules MC1 (**75**), MC2 (**78**), and MC3 (**71**) the results are encouraging and a little work would probably produce good results. Most likely the employment of a stronger base in the synthesis of MC2 would be enough to remarkably improve the reaction yield. A good result in MC2 synthesis would also open new synthetic routes for MC3, as the two molecules are very similar and could be synthesized from the same precursor. Further attempts could be made to optimize the proposed synthesis of MC3. The extension of the reaction time for the condensation step looks like the most promising direction to investigate. A completely new option could be to synthesize different model compounds, always tridentate to maintain the purpose of the research, but with different structures and based on other synthetic routes. One option could be to synthesize dibenzodioxocine rings or 4-O-5 bonds based molecules, and use them as central molecules for DHP polymerization. These naturally occurring unit linkages are natural branching points in *in vivo* lignin, and are exactly what our model compounds try to emulate. Once a proper synthetic pathway is found, it should be possible to mark or functionalize them in order to have distinguishable central molecules. Any tridentate molecule which cross couples with CA, in principle, could be a suitable model compound

for this research. The synthesis of these molecules would not only be relevant for this study: each tridentate model compound which positively interacts with CA could also be tested for DHP thin layers growth. As already said, a DHP thin layer could be very interesting from both a theoretical and an applicative point of view. Surface analysis of DHP could produce very meaningful information about its growth, while its modification could produce very useful materials, with tailored properties.

4.3.2 POLYMERIZATION

The purpose and goal of this research, instead of finding evidence for polymer chain loops, as expected, ended up being looking only for sign of CM-CA cross coupling. This change of goal caused some problems, because the experiments performed were designed for one purpose and used for another. For the future, we propose to proceed in a different way. Once a new CM will be ready for DHP polymerization, it is probably best to test its reactivity to CA (**29**), and only then to proceed with the proper DHP polymerization.

This could be accomplished with two experiments, differently designed, each for its purpose. During the first experiment, the CM in excess should be reacted with low amounts of CA, very diluted and very slowly added to the reaction mixture. These reaction conditions will maximize the CA-CM cross coupling and reduce the CA-CA coupling. Analysis of the CM-CA product will provide a reference and a starting point for the second polymerization experiment. This last should be performed with higher amounts of CA, in order to detect the closed loops. After the first reaction, it would be immediately understandable whether the tested molecule is worth the second step of the analysis or not. If the CM doesn't interact with CA, or if some other side reactions takes place, there will be no need to continue with the long, time and resources consuming series of polymerization experiments. With or without this procedure, future research will have to focus on the heavy fraction of CM DHP, and therefore face the problems of solubility and analysis resolution. The possibility to use other separation techniques, more reliable and precise than SEC, should be seriously investigated. The chance to perform HPLC or MS on both light and heavy samples could provide fundamental information. HPLC could separate simple CA coupling products from our products of interest, allowing us to analyze a purer sample. MS analysis of a pure sample could be precise enough to detect the presence of loops in the molecule, as two separate polymer chains will have a different Mw if they are linked together. Naturally, even a SEC experiment calibrated for higher Mw will

provide better information than the ones we gathered in this study. Another analytical tool that could be employed is solid state NMR. Even if the heavy fraction is usually at least partially soluble in DMSO, it is possible that this technique will produce more interesting results that the regular liquid phase NMR. Most of these techniques are delicate, and can produce results only under specific circumstances and conditions. It will be necessary to test if the machine requirements can be fulfilled by the samples. Once again the main obstacle will most likely be solubility.

Leaving the analytical problems aside, there is still much left to be done in the study of CM DHP. Two of the three model compound that were to be tested still have to be tried, the results produced by the MeRe (**72**) and $Ph(OH)_3$ (**76**) run need to be further investigated and maybe experiments with higher amounts of CA performed. There is a lot more to be done, but before moving forward with anything, it would be necessary to decide a strategy based on the information gathered by this study.

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Now that I think of it, I should also thank my family, but this thesis is in english so they probably won't read it. Thank you guys, just in case, you know I love you.

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